The Long Road Ahead: Understanding Road-related Threats to Reptiles and Testing if Current Mitigation Measures are Effective at Minimizing Impacts

by

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Thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science (M.Sc.) in Biology

School of Graduate Studies

Laurentian University

Sudbury, Ontario

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE

Laurentian Université/Université Laurentienne

School of Graduate Studies/École des études supérieures

Title of Thesis Titre de la thèse	THE LONG ROAD AHEAD: UNDERSTANDING ROAD-RELATED THREATS TO REPTILES AND TESTING IF CURRENT MITIGATION MEASURES ARE EFFECTIVE AT MINIMIZING IMPACTS
Name of Candidate Nom du candidat	Baxter-Gilbert, James H.
Degree Diplôme	Master of Science

Department/Program Département/Programme	Biology	Date of Defence Date de la soutenance	January 23, 2014

APPROVED/APPROUVÉ

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Abstract

Reptile populations are suffering substantial global losses and roads are identified as one of the leading threats to their persistence. Currently, efforts to mitigate this threat are being implemented with various levels of success. I studied the effectiveness of exclusion structures (*i.e.*, fencing) at preventing reptiles from gaining access to the road, and reducing road mortality. I also examined if population connectivity structures (*i.e.*, ecopassages) were effective at reducing habitat and population fragmentation and allowing individuals to access habitats, resources, and mates on both sides of a major road (4 lane highway). I found that the fence was ineffective at preventing reptiles from gaining access to the road; however, reptiles were observed using the ecopassages to cross the road. Behavioural trials testing painted turtles' (*Chrysemys picta*) willingness to use an ecopassage demonstrated that refusal was twice more likely than use of an ecopassage. I also examined the potential for roads to pose a physiological threat to roadside populations of reptiles by examining corticosterone (CORT), a stress hormone linked to negative health effects in cases of elevated levels over the long-term. To assess if individuals living near a major road had higher CORT levels than individuals from a less impacted population, I developed a novel means of measuring CORT from painted turtle claws in partnership with Dr. Gabriela Mastromonaco (Toronto Zoo). With long-term CORT levels considered as a proxy for chronic physiological stress, I did not find evidence that populations near roads had altered stress levels. However, this seminal study will provide the framework for further examination of more species, including species-at-risk, and a better understanding of effects of anthropogenic environments on wildlife health. As road ecologists strive to expand our understanding of the threats roads pose to reptiles, it is important that this field spans multiple disciplines, so that we can both understand the direct and indirect threats that roads cause and develop effective mitigation that preserves biodiversity within our anthropogenic landscape.

Acknowledgements

I would like to thank my two supervisors Dr. Jacqueline Litzgus and Dr. David Lesbarrères, not only for their mentorship during this degree, but also throughout my entire academic career. Both of you offered me nothing short of a tremendous learning experience filled with insight, encouragement, and support. Thank you for providing me with this opportunity, and for shaping me into a capable biologist able to undertake this challenge. I would also like to thank my other committee member Eric Cobb for your support with this project, and also your insights into matters of science, and environmental management and policy were all greatly appreciated. Thank you!

I would like to thank my grizzled field crew, you seasoned veterans you. Julia Riley, master and commander of the south recon unit. Jennifer Baxter-Gilbert, although not a biologist by formal training, you not only picked up the required skills for the job, but excelled at it (I am so proud of you). Chris Neufeld, the world of field biology anxiously awaits your return. Sean Boyle, starships were meant to fly… I'll see you in the doctor's lounge. Ron Maleau, we missed you the second year, but I cannot wait to see what you get up to after your schooling is done! Liv Monck-Whipp, this project was extremely lucky to have more than one MSc candidate working on it (I owe Dr. Fahrig a beer… or a case… or a brewery). Geoff Hughes, I think we may have to merge the song "On the road again" by Willie Nelson with "Harder Better Faster" by Daft Punk (thanks for coming out for another rodeo). All of you made this project work. Without you guys it would have been impossible. You are the dream team of road ecology. Thank you!

I would like to thank Magnetawan First Nation for all of their support for this research. Everyone from Chief and Council to all of the community members supported this work, and your hands-on help made all the difference. Jerry Smith, for starting the ball rolling on this partnership and keeping it going. Joe Noganosh, for always putting the critters first. Debbie and Bado, for supplying us with a place to live. You guys rock! William Diabo and Lloyd Noganosh, for continuing to support this project and helping us move it into the next phase. There are too many awesome people to name, everyone in the community deserves a massive thank you. From the bottom of my heart, Chi Miigwetch!

I would like to thank Dr. Gabriela Mastromonaco, Christine Gilman and Stacey Hayden for all of their hard work on the laboratory side of this study. Your wizardry is unmatched. Your methods, tests and protocols for extracting CORT from claws were amazing. I am glad I was able to help with the development of the smashy smashy technique. I am so pleased to have been able to partner with you. Thank you!

I would like to thank my labmates from the Litzgus and GEARG labs: Julia Riley, Matt Keevil, Kiyoshi Sasaki, Patrick Moldowan, Sean Boyle and Mike Colley. Herpington Manor and Co. for life! Also I would like to thank all of the volunteers that have helped on this thesis (there are way too many to list, but you know who you are). Thank you!

I would like to thank Laurentian University, the Ontario Ministry of Natural Resources, the Ontario Ministry of Transportation, NSERC, and Magnetawan First Nation for funding the project.

I would like to thank my parents, Bonita Baxter and Randy Gilbert. Thank you both for all of your love and support, and fostering my love of nature. To my friends and family, who have supported my love of reptiles and science (sorry for all of the special events and birthdays missed). I very much appreciate of all of your love and support.

Finally, I would like to say a particularly special thank you to Julia Riley. As the better half of “Team Awesome” it is difficult to find the appropriate words to thank you. I am so lucky that you decided to join me on this adventure. I could not have done it without you; your support, your insight, your brilliance, and your damned-determined passion for fieldwork made this project a success. I cannot wait to see what our next adventure brings.

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General Introduction

Conservation of biodiversity

The planet is losing biodiversity at a rate much faster than ever previously recorded (Rockström et al. 2009). The Quaternary extinction began approximately 50,000 years ago, and there has been a dramatic decrease in biodiversity during this time (Koch & Barnosky 2006). The natural background rate of extinction is estimated to be between 0.1-1 species per million species per year, but the current extinction rate is thought to be 10-1000 species per million species per year (Rockström et al. 2009). The Quaternary extinction is unique because its causes (*e.g.*, over-exploitation, invasive species introduction, habitat degradation, and climate change; Venter et al. 2006) are directly linked to a single species, humans (Randall 1991; Burney and Flannery 2005; Koch & Barnosky 2006). There is strong evidence that within only a few hundred years from first contact with humans, an ecosystem will suffer a significant loss of biodiversity (Burney and Flannery 2005).

Reptiles, as a taxon have suffered substantial losses (Gibbons et al. 2000; Böhm et al. 2013). For example within Ontario, Canada, 19 of the 26 reptile species (73%) are listed as Species-at-Risk (SAR) with anthropogenic activities being the predominate threat (Seburn & Seburn 2000; Venter et al. 2006; COSEWIC 2011). Similar to other developed nations, Canada's reptiles are imperilled by various anthropogenic activities, such as: habitat destruction (with 84% of Canada's imperilled species listing this as a threat), introduced invasive species (56%), pollution (41%), persecution and over-harvesting (39%) and climate change (27%; Venter et al. 2006; McCune et al. 2013). Not surprisingly, habitat destruction is the leading cause of reptile decline across the most populated Canadian province, Ontario (Venter et al. 2006). Since the 1800s, much of southern Ontario's landscape has been dramatically altered by deforestation, wetland destruction, and urbanization (Hecnar & M'Closkey 1996). Unfortunately, southern Ontario is home to half of the reptile biodiversity in Canada (Seburn & Seburn 2000; COSEWIC 2011). It should be a goal of ecologists, conservationists, government agencies, and land-use planners to ensure that the biodiversity losses seen in southern Ontario are not replicated as the human-footprint encroaches into central Ontario, and the rest of Canada.

The effect of roads on wildlife

Of many types of habitat destruction, road construction/expansion presents persistent and enduring threats, and greatly contributes to the imperilment of many SAR (McKinney 2002). Throughout central Ontario, Highway 69 is being expanded into a large multilane 400 series highway (Pagnucco 2005). Highway 69 is the main roadway connecting northern and southern Ontario. In 2008, this highway had an annual average daily traffic volume (AADT) of 7,600 vehicles at the four-lane section near Parry Sound, Ontario (Ministry of Transportation Ontario 2008). It is expected that the traffic volume will increase with further urbanization of central Ontario, prompting the need for an expanded highway (Pagnucco 2005). This highway also bisects one of Ontario’s richest areas of reptile biodiversity, the Georgian Bay coastline (Hecnar et al. 2002). Mirroring the developmental history of southern Ontario, urbanization and human development is spreading into a reptile biodiversity hotspot. Consequently, the expansion of Highway 69 into a 400 series highway will impose a long-lasting and significant threat to SAR reptiles inhabiting the Georgian Bay coastline (Rouse & Willson 2001; McKinney 2002).

Road mortality and Ontario reptiles

Road mortality refers to point source mortality that is associated with roads and traffic, and it is listed as a threat for 31% of Canada's imperilled species (McCune et al. 2013). Similarly, roads are a leading cause of decline for all three orders of reptiles (*i.e.*, turtles, snakes, and a lizard) within Ontario (Ashley & Robinson 1996; Seburn & Seburn 2000; COSEWIC 2007, 2011). Turtles are imperilled by roadways due to their long-distance seasonal movement patterns, and the tendency for gravid females to select road shoulders as nest sites (Gibbs & Shriver 2002; Marchand & Litvaitis 2004; Steen et al. 2006). Nesting on roadsides drastically increases the amount of time a turtle is exposed to traffic, as nesting takes a longer time than a road crossing. Also, a recent study showed that 2.7% of drivers will deliberately aim to hit a turtle with their vehicle (Ashley et al. 2007). With an expected AADT of over 10,000 vehicles per day along Highway 69/400 (Pagnucco 2005), approximately 270 drivers per day could thus deliberately aim to hit turtles if they encounter one. In another study along a 3.3 km stretch of the Long Point Causeway in southern Ontario, 160-202 turtles were killed annually after colliding with motor vehicles (Ashley & Robinson 1996). This study demonstrated that when an active highway with no mitigation measures bisects an area of high reptile biodiversity and abundance, there is a corresponding high level of reptile road mortality.

Adult mortality poses a great threat to turtle populations, because in order to sustain healthy populations it is essential to maintain adult survivorship to offset naturally low juvenile recruitment and delayed sexual maturity (Gibbs & Shriver 2002). For many healthy populations of turtles, nest depredation and juvenile death are the primary sources of mortality (Bowen & Janzen 2005). A study done on snapping turtles (*Chelydra serpentina*) revealed that, 30 to 100% of nests were depredated annually, with an average of 70% that were depredated in any given year (Congdon et al. 1987). Of the remaining, un-predated nests (average of 30% of the total nests laid each year), only 22% of the hatchlings were estimated to survive the first year (Congdon et al. 1987). Overall, this amounts to only 6% of the eggs laid in a snapping turtle population naturally surviving to one year of age (Congdon et al. 1987). As there are naturally high rates of mortality in the embryonic and juvenile life stages, and delayed sexual maturity, turtles rely on high adult survivorship and long-term repeated reproductive activity to sustain healthy populations (Tinkle et al. 1981; Congdon et al. 1994; Litzgus 2006). This life history strategy is referred to as 'bet-hedging' (Stearns 1976). If adult mortality rate increases, this strategy is no longer is effective (*e.g.*, if annual mortality rate of adult snapping turtles exceeds 1%, the population will decrease by half in 20 years; Congdon et al. 1994). Therefore, if adults are being killed on roads during their seasonal movements (*e.g.*, from hibernation sites to foraging sites, nesting migrations, etc.), populations drastically decline as the 'bet-hedging' life history strategy is disrupted (Dodd et al. 2004; Seburn 2007; Andrews et al. 2008).

Snake populations are also imperilled by roads (Rouse & Willson 2001; Rowe et al. 2007; Shepard et al. 2008). In fact, many Ontario SAR snakes (*e.g.*, eastern hog-nosed snake; *Heterodon platirhinos*, massasauga rattlesnake; *Sistrurus catenatus*) have been documented crossing roads during their seasonal movements, resulting in mortalities (Rouse et al. 2011). Road crossings put snakes directly into harm, and as with turtles, approximately 2.7% of drivers will intentionally attempt to run over snakes (Rudolph et al. 1998; Ashley et al. 2007). Snake species have different interspecific behavioural responses to roads (Rouse et al. 2011; Andrews & Gibbons 2005). Certain species, such as garter snakes (*Thamnophis* sp.), rattlesnakes (*Crotalus* sp. and *Sistrurus* sp.), and watersnakes (*Nerodia* sp.) will cross using an angle perpendicular to the road, minimizing the time spent on the road surface (Shine et al. 2004;

Andrews & Gibbons 2005). Other species differ in their road crossing pace, resulting in greater vulnerability to road mortality for slower-moving species (Andrews & Gibbons 2005). Additionally, snakes use the road surface to bask during the day and to absorb radiant heat at night (Enge & Wood 2002). This use of the road for thermoregulation increases mortality risk, and may be a cause of population declines (Rudolph et al. 1998; Rouse et al. 2011). Basking differs from road crossing due to the amount of time spent on the road and while most snake species minimize time spent on the road surface during a crossing (Shine et al. 2004; Andrews & Gibbons 2005), road or roadside basking may constitute threat over a long time period (Rosen & Lowe 1994; Enge & Wood 2002).

The five-lined skink (*Plestiodon fasciatus*) is the only lizard native to the province; it is also a SAR of Special Concern (COSEWIC 2011). Within its global range it is also imperilled by roads (COSEWIC 2007) and while much of the documentation of road mortality is from the United States (Aresco 2005; COSEWIC 2007), roads have also been noted as source of mortality for skinks in southern (Hecnar & Hecnar 2005; COSEWIC 2007) and central Ontario (Baxter-Gilbert et al. 2013). In central Ontario skinks living around roads have been seen to select thermally unsuitable habitats, possibly in favour of increased prey abundance, and actively engage in road crossing resulting in mortality (Baxter-Gilbert et al. 2013). Although discussion of population-level threats to this species from roads is absent from the literature, the presence of this lizard near and on roads, with known negative effects on individuals, suggests further investigation is required.

Reptile population and habitat fragmentation by roads

Although road mortality presents the most obvious threat to reptile populations, the other major known threat posed to reptiles by roads is population and habitat fragmentation (Shepard et al. 2008; Clark et al. 2010). Due to interspecific differences in the willingness to cross roads, interspecific differences exist in how significant a threat habitat fragmentation is to populations. Such differences may be based on differences in crossing ability (based on crossing angle and speed; Andrews & Gibbons 2005), and crossing success (varying from 2-30%; Aresco 2005). Fragmentation affects populations over two distinct time scales: immediate, and long-term. The immediate threats posed by fragmentation is the inability for individuals within populations divided by a road to access important habitats (*e.g.,* foraging sites, nesting sites, overwintering sites, etc.) which may reside on the opposite side of the road (Fahrig & Rytwinski 2009). The long-term effects of fragmentation result in isolating individual sub-populations from one another (Fahrig & Rytwinski 2009). The effects of fragmentation by roads on population genetics have been examined in a number of species (*i.e.*, amphibians and mammals); however, genetic effects on reptiles have not yet been examined (Holderegger & Di Giulio 2010; Beebee 2013). One of the obvious issues with studying the effects of fragmentation on reptile population genetics is the large time-lag required to measure any effect; for some species, such as watersnakes, a minimum time-lag of 10 years would be sufficient (Rowell 2012), whereas a minimum time-lag for snapping turtles would be 50 years (Congdon et al. 1994). Yet, collection of baseline population genetics and building genetic databases in areas with roads will be required to eventually understand the implications of long-term fragmentation, and road mortality mitigation, on reptile populations.

Mitigation measures to offset road mortality and fragmentation

In order to respond to the direct negative effects roads pose to reptile populations, mitigation measures are currently being installed into specific roadways where identified SAR reptile populations and habitats are bisected by major roads (*e.g.,* Highway 69/400 in central Ontario; Gunson et al. 2009). It is proposed that the most effective road mitigation measure is to implement a series of exclusion structures in conjunction with population connectivity structures in areas where reptiles frequently encounter high traffic roads or dense road networks (Dodd et al. 2004; Aresco 2005; Ashley et al. 2007). The purpose of the exclusion structures (*e.g.,* fences or barriers) is to restrict an individual reptile's access to the road surface and funnel individuals attempting to crossing towards connectivity structures (Dodd et al. 2004; Aresco 2005), in turn the habitat and population connectivity structures (*e.g.,* culverts, overpasses, underpasses, or ecopassages) are designed to allow individuals to gain access to habitat and conspecifics on both sides of the road (Dodd et al. 2004).

When discussing mitigation measures it is important to determine what the goal of mitigation is, whether it is to eliminate all or some of the threats posed by roads, or to simply lessen the impacts, and also determine if the mitigation is effective. Furthermore, we can classify effectiveness several ways: 1) a general reduction in either the number of threats or the severity of threats, 2) a statistical reduction in either the number of threats or the severity of threats, or 3) a biologically significant reduction in both the numbers of threats and the severity of threats so that local populations of target species are no longer at risk of extinction due to the presence of roads and traffic. Recently there has been a sharp increase in the amount of roadway mitigation projects; however, the effectiveness of these mitigation structures has not been thoroughly quantified (Forman et al. 2003; Jochimsen et al. 2004; Lesbarrères & Fahrig 2012). In many road ecology studies, the research rarely extends past a presence/absence study during the before and after mitigation periods, and sometimes only consists of a survey after mitigation is in place. More rigorous studies (*i.e.*, using hypothesis-based experimental methods), must be used to determine the effectiveness of mitigation structures (Forman et al. 2003; Jochimsen et al. 2004; Lesbarrères & Fahrig 2012). Moreover, lack of sufficient assessment poses an issue for conservation, as the allocation of funding to protect SAR is finite and must be spent effectively (Mahoney 2009). It is thus crucial to test the effectiveness of mitigation measures to both address threats to SAR, and to develop recommendations to ensure mitigation measures can be regularly built into roadways (Roedenbeck et al. 2007; van der Ree et al. 2007; Glista et al. 2009). In the first chapter of my thesis, I present an evaluation of the effectiveness of road mitigation structures (population and habitat connectivity structures, and exclusions structures) that are currently used on a section of highway, so that both successes and failures can be identified for inclusion or modification in future projects.

Examining unknown secondary negative effect of roads

An understudied aspect of road ecology is the investigation of potential negative physiological effects on individuals or populations living in proximity to major roadways. Few studies have addressed the physiological effects of roads on wildlife (Crino et al. 2011, Morgan et al. 2012), and even fewer have studied these effects in reptiles (Andrews et al. 2008). The effects of chemical, light, and noise pollution may have long-term effects on reptile populations, and these effects can be expressed as chronic physiological stress and may even have negative fitness implications that manifest as further population declines (Angelier & Wingfield 2013; Jessop et al. 2013). An individual's response to a stressor typically results in a biochemical reaction that prepares the individual for a noxious stimulus through the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Romero 2004), and this involves the allocation of energy away from long-term physiological functions and into short-term physiological functions (Romero 2004; Busch & Hayward 2009). A common example is the allocation of energy away from digestive and reproductive functions, and funneling it into priming muscles during a flight or fight response (McCarty 2007; Adamo 2012). Chronic physiological stress has negative effects on individuals through the suppression of long-term physiological functions, such as reproduction, growth, and immune functions (Cabezas et al. 2007; Cyr & Romero 2007). Examining stress levels in turtles around a major roadway is the focus of my second thesis chapter. This chapter has two objectives: 1) to develop a simple and non-invasive method to evaluate chronic physiological stress in turtles, and 2) to test if turtles living in proximity to roadways display higher stress levels compared to stress levels of individuals in a less impacted area.

Significance

The overall goals of my thesis are to address the threats roads pose to reptile populations in central Ontario, and develop a means to rigorously measure and mitigate direct and indirect road threats. It is crucial to be able to assess road threats, and effectively mitigate those threats in order to effectively recover and conserve SAR reptiles. Evaluation and the creation of novel assessment techniques is necessary for adaptive management, so that we can move successful measures forward, and incorporate them into areas where reptile populations are threatened. However, for adaptive management it is equally important to identify failures in mitigation so they can be solved before those methods are implemented elsewhere. With a greater understanding of both successes and failures, there is a much greater chance at reducing, or eliminating, the devastating effects roads have on reptile populations. Furthermore, it is critical to look at the non-obvious threats (*e.g.*, subtle negative physiological effects) so that the full spectrum of road threats can be identified and eventually mitigated. Rigorous research and adaptive management is the only chance we have to properly slow the tide of species loss, especially in a group that is extremely imperilled – reptiles. Objectives of herpetological research should always relate to conservation, otherwise we are just paleontologists in the making (F. Cook pers. comm.).

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Chapter 1

A hard look at the road ahead:

A comprehensive examination of the effectiveness of mitigation for reducing reptile road mortality while maintaining population connectivity

Abstract

Roadways pose serious threats to animal populations. The installation of roadway mitigation measures is becoming increasingly common, yet studies that rigorously evaluate the effectiveness of these conservation tools remain rare. A recent highway expansion project in central Ontario, Canada included a series of mitigation measures designed to offset detrimental effects on threatened reptile species. I used a Before-After-Control-Impact study design to compare reptile abundance on the highway before and after mitigation at a test site and a control site. At both locations, 13 km of road were surveyed for live and dead reptiles by car three times per day and on foot once per day from 1 May to 31 August in 2012 and 2013. Radio telemetry, automated microchip readers, and wildlife cameras were used to monitor reptile movements and use of ecopassages. Additionally, a willingness to utilize experiment was conducted to quantify turtle behavioral responses to ecopassages. I found no difference in abundance of turtles on the road between the un-mitigated and mitigated highways, and an increase in mortality rates for both turtles and snakes post-mitigation, suggesting that the fencing was not effective at keeping animals off the road. Although ecopassages were used by reptiles, the number of crossings through ecopassages was lower than crossings on the highway, and turtle willingness to use ecopassages was lower than that reported in previous arena studies. My rigorous evaluation of roadway mitigation demonstrated that when exclusion structures fail, the effectiveness of population connectivity structures is compromised. My project emphasizes the need for quantitative evaluation of road mitigation to allow for adaptive management and optimization of these increasingly important conservation tools for numerous imperilled taxa.

Introduction

Increasing rates of urbanization and resource demands, with associated habitat destruction and fragmentation, have led to the imperilment of much the world's biodiversity (Forman & Alexander 1998; McKinney 2002). Roads and traffic may present some of the longest lasting effects from both point-source mortality and enduring habitat and population fragmentation (Forman & Alexander 1998; Trombulak & Frissell 2000). The threats posed by roads extend from individual mortality to population-wide effects, as barriers within populations can lead to loss of genetic diversity and isolation (Holderegger & Di Giulio 2010). Over the last two decades the field of road ecology has grown to include examination of numerous taxa and incorporates a wide variety of disciplines, with the common goal to better understand the interaction between roads and wildlife (Forman & Alexander 1998; Fahrig & Rytwinski 2009; van der Ree et al. 2011; van der Grift et al. 2013). Closely tied to this research effort is the development of mitigation strategies aimed at protecting wildlife from the negative effects of roads (Huijser et al. 2007), with particular focus on imperilled species (*e.g.,* Florida panther, *Felis concolor coryi*, Foster & Humphrey 1995; desert bighorn sheep, *Ovis canadensis nelsoni*, Epps et al. 2005; long-toed salamanders, Ambystoma *macrodactylum*, Pagnucco et al. 2011). Reptiles, considered one of the most imperilled groups of animals globally (Böhm et al. 2013), have been particularly affected by the proliferation of roads (Gibbs & Shriver 2002; Row et al. 2007; Andrews et al. 2008), and conservation strategies to minimize threats posed by roads are becoming common (Dodd et al. 2004; Aresco 2005).

Threats to reptiles from roads, as with all effected taxa, are multifaceted and often relate to specific ecological and life-history traits, behaviours, and movement patterns (Forman et al. 2003; Andrews & Gibbons 2005; Gibbs & Steen 2005). Turtles regularly encounter roads during long-distance seasonal movements and road mortality of adults leads to population declines because of the 'bet-hedging' life history of turtles that requires high adult survivorship for population persistence (Heppell 1996; Gibbs & Shriver 2002; Marchand & Litvaitis 2004). Turtles are particularly susceptible to road mortality, with mortality rates as high as 98-100% of individuals killed during their first road crossing attempt (Aresco 2005). Similar to turtles, seasonal movements of snakes also require road crossings, and road mortality has been identified as a population-level threat to several species (Clark et al. 2010; Rouse et al. 2011). Snakes also bask on the road surface during the day, and absorb radiant heat at night; these behaviours prolong exposure to traffic and increase the likelihood of collisions (Rosen & Lowe 1994). Furthermore, road threats are amplified by an average of 2.7% drivers who will intentionally run over reptiles (Ashley et al. 2007).

Mitigation measures to reduce negative effects of roads have been designed to reduce road mortality by installing exclusion structures and reduce fragmentation by installing population connectivity structures (Dodd et al. 2004; Aresco 2005; Ashley et al. 2007). The integration of such structures into highway designs is becoming increasingly common, yet the effectiveness of these mitigation measures is rarely quantified (Forman et al. 2003; Jochimsen et al. 2004; Lesbarrères & Fahrig 2012; van der Grift et al. 2013). This lack of assessment is an issue as the availability of conservation funding is limited and thus must be allocated efficiently (van der Grift et al. 2013). Rigorous assessments of mitigation are required so that a framework for effective mitigation that can be regularly implemented into roadways (Roedenbeck et al. 2007; van der Ree et al. 2011; Glista et al. 2009).

Successful evaluation of roadway mitigation measures must examine the following criteria (Little et al. 2002; Forman et al. 2003; Jochimsen et al. 2004): 1) reduction in road mortality, 2) maintenance of habitat connectivity and access to critical habitats, 3) maintenance of dispersal routes (*e.g.*, daily and seasonal) and continuity of metapopulation processes, 4) prevention of prey-trap formation, 5) preservation of habitat quality, including minimization of habitat loss, and 6) preservation of gene flow throughout populations. My study quantitatively assessed these criteria in order to evaluate the effectiveness of mitigation structures along a major roadway. My study directly addressed criteria 1 to 4, and we gathered genetic baseline data for future projects to address criteria 5 and 6. I rigorously assessed the effectiveness of the mitigation via 6 methods: i) Before-After-Control-Impact (BACI) study to examine change in reptile abundance on roads, ii) radio-telemetry to examine reptile movements around roads, iii) camera traps in ecopassages to determine reptile and predator presence, iv) implanting reptiles with passive integrated transponders (PIT tags) and installing automated readers in an ecopassage to determine reptile use, v) a willingness to utilize (WTU) experiment to assess likelihood of ecopassage use, and vi) the creation of a pre-mitigation genetic baseline for future analysis. If the exclusion structures are effective at preventing reptiles from accessing the highway, I expect a significant decrease in the abundance of reptiles on the highway post-mitigation (criteria 1; method i). Concurrently, if the connectivity structures are effective at promoting population and habitat connectivity, then I expect that individuals should use the ecopassages to gain access to resources on either side of the highway (criteria 2-6; methods ii-iv).

Methods

Study area and mitigation measures

The mitigation measures were constructed along a newly expanded section of Highway 69/400 in central Ontario, Canada. This major thoroughfare bisects one of Canada's richest areas of reptile biodiversity, the Georgian Bay coastline (Hecnar et al. 2002), an area with a high number reptiles designated as species at risk (SAR; COSEWIC 2011). The highway expansion and associated increase in traffic present long-lasting and significant threats to 6 species of turtles (5 SAR) and 12 species of snakes (5 SAR) in the region (COSEWIC 2011).

My study was conducted at two sites located 50 km apart: (1) the impact site, near Burwash, Ontario, Canada and (2) the control site, at Magnetawan First Nation. The survey area at both sites consisted of a 13 km section of the highway within comparable habitat. The impact site was a 2-lane un-mitigated highway during 2012 and a 4-lane mitigated highway in 2013. During construction of the mitigation measures and realignment of the highway, traffic was redirected from the original Highway 69 to the new Highway 69/400 (0.5 km east) on 6 June 2012. Since the mitigation was not complete after the active season (Fall 2012) and there was no difference in reptile abundance after the redirection (Mann-Whitney-Wilcoxin test, for turtles $W_1 = 13707$, $P = 0.72$; for snakes $W_1 = 13648.5$, $P = 0.79$) I felt confident in classifying the new alignment as comparable to the original Highway 69. The control site, a 2-lane highway, remained un-mitigated during both study years.

The mitigation measures at the impact site consisted of an exclusion structure (*e.g.*, reptile fencing) and three population connectivity structures (*e.g.*, ecopassages). The reptile fencing consisted of a heavy gauge plastic textile extending 0.8 m above- and 0.2 m below-ground with a 0.1 m wide lip running perpendicular underground. The fence was affixed to the base of a 2.3 m tall page-wire fence intended to keep large mammals off the highway (Fig. 1 A, B). Sections of reptile fencing were installed along the impact site in areas that were identified to be potential hotspots for reptile road crossings. The reptile fence connected the three ecopassages (spaced 450-600 m apart), and extended beyond the north ecopassage by 600 m and beyond the south ecopassage by 150 m. Each ecopassage consists of two 3.4 m x 2.4 m x 24.1 m concrete box culverts that cross the north-, and south-bound lanes of the highway (Fig. 1 C). A fenced 15.3 m gap connects each culvert below the north-, and south-bound lanes (Fig. 1 D), allowing light to enter the ecopassages (Woltz et al. 2008). All field work involving animals adhered to the guidelines of the Canadian Council on Animal Care and an approved Laurentian University Animal Care Committee protocol (AUP# 2013-03-01).

Method i: BACI study

The BACI study examined the differences in abundance of reptiles on the highway between the Before (2012) and After (2013) periods. Vehicular surveys were conducted at each of the impact and control sites (both 13 km of highway) at simultaneous time intervals (09:00, 18:00, 22:00) daily from 1 May to 31 August each year. Roadside walking transects (RST) were conducted on foot and covered 2 km of the highway within the full 13 km of the study site. At the impact site, the RST was located in an area scheduled for mitigation in 2012 and mitigated 2013. The RST at the control site was selected to incorporate comparable habitats to those of the impact site RST. The RSTs were walked after the 09:00 vehicular survey each day (at approximately 10:00).

During all survey types, the location, behaviour, mortality status and morphometric data were recorded for any reptile found on the highway. When measuring abundance of reptiles, morality status was not separated as both living and deceased reptiles represent failures in the exclusion structure. To determine if reptiles were being recaptured, each live reptile captured on the highway was individually marked. Turtles were notched in the marginal scutes (Cagle 1939), while snakes were marked with a permanent marker. During the 2013 field season, living snakes and turtles found at the impact site were also marked with a subcutaneously implanted PIT-tag. After processing, individuals were released at a safe distance off the highway in the direction they were heading, to reduce the likelihood of immediate contact with traffic. If the reptile was deceased, the individual was removed from the road to avoid being re-counted.

Differences between reptile abundances on the highway between Before and After periods were examined using a BACI analysis of variance (ANOVA; Smith 2002) and a Poisson generalized linear model (GLM) for non-parametric count data. Both tests included the fixed effects of period (Before, After), site (Control, Impact), and the interaction between these two effects (Smith 2002). The difference in abundance of snakes and turtles between the two sites was also compared between Before and After periods by a two-sample t-test for unequal variances (Smith 2002).

Method ii: Radio telemetry

The spatial ecology of two species of turtles occurring at the impact site was studied using radio-telemetry. During both the 2012 and 2013 active seasons, adult Blanding's turtles (*Emydoidea blandingii;* N=10, threatened, COSEWIC 2011) and snapping turtles (*Chelydra serpentina;* N=12, special concern, COSEWIC 2011) were captured within 1 km of the highway and outfitted with radio transmitters. Individuals were tracked every 2 to 3 days; locations were recorded using a handheld GPS unit, and specific behaviours and habitats were recorded.

Home range sizes (95% minimum convex polygons, MCPs; Litzgus and Mousseau 2004), and the number of highway crossings per individual were calculated using ArcGIS 10.0 (ESRI, 2011). Minimum effort was calculated to be 35 track days to achieve an accurate estimation of 95% home range, and only individuals fitting this amount of track effort from 2012 and 2013 were used. I compared home ranges size between the Before and After period using a two-tailed, paired t-test (turtle's home ranges were pooled, N = 4 snapping turtles and N = 4 Blanding's, as no difference was found between species; t_7=2.18, P=0.82). As well, I noted if individual home ranges overlapped with the highway post-mitigation for all radio-tracked turtles. These spatial variables were averaged for each species across both years, and data are reported as means, followed by ± SE.

Methods iii & iv: Ecopassage monitoring

Wildlife cameras (TrophyMAX, Bushnell) were installed in both entrances of the ecopassages to monitor use by reptiles and potential predators (Dodd et al. 2004, Pagnucco et al. 2011). Cameras were mounted to the ceiling of the culvert, and aimed directly at the ground to maximize frame coverage. To increase capture success during the night, as reptiles are ectothermic and thus less likely to trip the infrared sensor, cameras were programmed to take a photograph every minute between the hours of 18:30 to 6:30 (Pagnucco et al. 2011), while the motion sensor trigger activated the cameras for the other 12 hours per day. Pagnucco et al. (2011) noted that combining the motion sensor trigger with fixed-time photography increased detection, yet still only captured 56% of the individuals crossing.

Before the 2013 field season, automated PIT-tag readers (HPR, Biomark) with loop antennas (BIO 10 Antenna, Biomark) were installed in the center ecopassage at the impact site.

The antennas spanned the entire entrance of the ecopassage, preventing individual from crossing without entering the detection field. The readers constantly scanned and logged the PIT-tag number of any animal that passed through the loop antenna, and the date and time of crossing. During the 2013 active season, adult turtles (N=38: 6 painted turtles, *Chrysemys picta*; 15 Blanding's turtles; 17 snapping turtles) and snakes (N=20: 8 eastern gartersnakes, *Thamnophis sirtalis;* 12 northern watersnakes, *Nerodia sipedon*) found within 1 km of the highway were captured and outfitted with a subcutaneously-injected PIT-tag.

Method v: Willingness to utilize (WTU) experiment

During the 2013 active season, painted turtles (N=54) were collected by hand or with a dip-net from a wetland 2.5 km west of the highway. Individuals were transported by car to a testing site at the east entrance of an ecopassage. Painted turtles were used as the model organism for the WTU experiment because of their ability to orient using the sun (DeRosa & Taylor 1978; Caldwell & Nams 2006). This ability allowed us to create a scenario in which a turtle would want to cross the highway to return to its known home range (Ernst 1970). The ecopassage was located between the individual and its home wetland, thus simulating a turtle making a seasonal movement to critical habitat (*e.g.,* overwintering or nesting sites).

Prior to the experiment, individuals were temporarily outfitted with a radio transmitter with tape, and placed in an acclimation box 5 m from the entrance of the ecopassage. The turtle was left to acclimate to the sun's position, the substrate, and the noise and smell of the highway for 10 min. After the acclimation period, the box was remotely opened by a researcher situated behind a blind (Andrews & Gibbons 2005). Turtle movements were monitored from behind the blind to assess the individual's interactions with the ecopassage. Each individual turtle's behaviour was ranked using a measure of crossing success on a scale from 0-5: 0) not willing to use, walked away; 1) made no choice, remained at entrance; 2) experimenting with use, entered first quarter of culvert; 3) cautiously willing to use, crossed half of culvert; 4) slowly willing to use, crossed three quarters of culvert; and 5) completely willing to use, crossed full length of culvert (Lesbarrères et al. 2004). After 20 min, or if an individual moved greater than 10 m out of the testing area, the turtle was collected and its location was recorded. All WTU tests occurred within less than 8 h of capture. After the WTU test, the turtle was marked to avoid re-capture, and was returned to the original site of capture within 12 hours.

A Pearson chi-squared test was used to compare the number of turtles that (a) refused to use the ecopassage (score 0), (b) turtles that made no decision regarding the ecopassage (score 1) and (c) turtles willing to use the ecopassage (scores of 2-5); to the results of 30 minute trials in artificial arenas as recorded by Paulson (2010): (a) 91/190 (48%) refused, (b) 10/190 (5%) no decision, and (c) 89/190 (47%) willing to use ecopassages.

All statistical tests were conducted in R (version 2.15.0, R Development Core Team 2012). All summary data are reported as means followed by 1 SE. The significance level of α = 0.05 was used for all statistical tests.

Method vi: Genetic sampling

Genetic samples (*i.e.*, blood and tissue) were taken from living and deceased reptiles found on the highway, and within 1 km of the highway, at the impact site. The genetic material was stored on nucleic acid storage cards (FTA, Whatman), and information on species, date, location, and gender was recorded and the cards archived. Samples were collected during both years from adult reptiles.

A total of 11 species were sampled (N=176 individuals), and for 4 species (painted turtle, snapping turtle, gartersnake and watersnake) I collected over 30 individual samples. The generational time of each species differs; a minimum of 10 years would be needed to test genetic isolation for northern watersnakes (2-3 year generation time, and a 7-9 year life span; King & Lawson 2001; Rowell 2012), while a minimum of 50 years would be necessary to test for alleviation of genetic isolation post-mitigation in snapping turtles (15-25 year generation time, and a 60-100 life span; Congdon et al. 1994). No results for this method will be reported herein as this dataset will serve as a baseline for a future population genetic study to assess the effectiveness of the mitigation.

Results

Method i: BACI study

A total of 960 road surveys were conducted in 2012, and 974 were conducted in 2013, resulting in a combined total of 618 snakes and 378 turtles being recorded on the highway at both sites (Table 1.1; Fig.1.2, 1.3). The average mortality rate (no. dead out of total no. on the road) across both sites and years was 82.6%. More specifically, the mortality rate at the impact site increased from 69.5% to 89.5% for turtles, and from 65.4% to 90.0% for snakes between the Before and After periods.

The BACI ANOVA indicated no significant interaction between period (Before and After) and site (Control and Impact) for turtle abundance ($F_{1,488\,=}$ 0.38, P = 0.54) although there was a significant interaction for snakes ($F_{1,488\,=}$ 13.24, P < 0.01). Similarly, the Poisson GLM demonstrated no significant interaction between period (Before and After) and site (Control and Impact) for turtles ($z_{488\,=}$ -0.05, P = 0.57) but there was a significant interaction for snakes ($z_{488\,=}$

3.60, $P < 0.01$). Relative turtle abundance (Control relative to Impact) on the highway did not differ between the Before and After periods ($t_{242} = -0.69$, $P = 0.49$). In contrast, relative snake abundance on the highway decreased between the Before and After periods ($t_{226} = 3.90$, $P < 0.01$). The results of all of the tests corroborate that there was no difference in daily turtle abundance on the highway between the Before and After period (Fig. 1.4 A), while there was a difference in daily snake abundance on the highway between the Before and After period (Fig. 1.4 B); this difference did not demonstrate a reduction in snake abundance at the impact site, but rather the prevention of an increase.

Method ii: Radio telemetry

Home range sizes did not differ between the Before (Fig. 1.5) and After (Fig. 1.6) period ($t_7 = 2.36$, $P = 0.83$), allowing the homes range sizes to be pooled from both periods. Blanding's and snapping turtle average home range size was 43.1 ± 23.5 ha and 48.9 ± 10.4 ha, respectively. Pre-mitigation, there were 4 road crossings by 4 radio-tagged turtles (2 Blanding's turtles and 2 snapping turtle). Post-mitigation, there were 11 road crossings by 3 radio-tagged turtles (2 snapping turtles, and 1 Blanding's turtle; Fig. 1.6) and 1 snapping turtle passed through the exclusion fence but did not cross the highway (Fig. 1.7).

Methods iii & iv: Ecopassage monitoring

A total of 485 individual animals were photographed in the ecopassages, consisting of at least 23 non-reptile and 3 reptile species. Waterfowl (present in 40.2% of photographs) was the most common taxon recorded using the ecopassages, and reptiles were one of the least photographed taxa (in 2.0% of photos). Painted turtles were photographed in the ecopassages on

6 occasions (4 adults and 2 hatchlings; 1.2% of photos). An adult snapping turtle (0.2% of photos), and 3 northern watersnakes were also photographed in the ecopassages (0.6% of photos). A number of reptile predators were also seen using the ecopassages; Great Blue Herons (*Ardea herodias*; 8.9% of photos), raccoons (*Procyon lotor*; 8.2% of photos), American minks (*Neovison vison*; 2.3% of photos), and coyotes (*Canis latrans*; 1.7% of photos). Additionally, during regular camera maintenance, snapping turtle tracks were observed that were not associated with a photograph, and a live juvenile red-bellied snake (*Storeia occipitomaculata*) was also observed within an ecopassage.

Although frequent tests of the automated PIT-tag reader occurred, only two PIT-tagged animals were recorded in the ecopassage: a watersnake and a painted turtle entered the ecopassage. In both cases, the readers did not detect a complete crossing (*i.e.*, PIT-tags were not logged at both entrances), rather it appears that the individuals either retreated from the entrance of the ecopassage after approaching the reader, or that they circumvented the exclusion fencing within the highway median between the ecopassages.

Method v: Willingness to Utilize (WTU) Test

Most turtles did not make a decision regarding use of the culvert within the allotted time (N=37/54; 69%). Of the remaining individuals (N=17/54; 31%), more than twice as many turtles refused to use the culvert (N=12/17; 71%) than were willing to enter (N = 5/17; 29%). When my results were compared to those obtained in an arena setting (Paulson 2010), I found that far fewer individuals were willing to use an ecopassages below an active highway as compared to in an arena (X^2_2 = 863.52, $P < 0.001$).

Discussion

Effective road mitigation relies on the ability of structures to both exclude individuals from the road surface, as well as structures that connect individuals to resources and conspecifics across the road. The success of the connectivity structures is intrinsically reliant on the success of the exclusion structures. My findings suggest that the materials and design of the exclusion structures used in this study are prone to failure compromising the effectiveness of the entire system.

Effectiveness of the exclusion structures

The fencing was ineffective at preventing turtles from gaining access to the road surface, and turtle mortality rate increased by 20% in the post-mitigation period. By contrast, the fencing reduced the relative abundance of snakes on the road but this reduction was not because of a decrease in the abundance of snakes on the highway at the impact site per se, but rather because the fence likely prevented the large increase in snake abundance observed at the control site in 2013 (Fig. 1.4 B). Furthermore, there was a 25% increase in snake mortality at the impact site post-mitigation, indicating that although fewer snakes were on the highway, many more were killed than during the pre-mitigation period. The increase in mortality rate observed in both turtles and snakes post-mitigation may be attributed to a corralling-effect of the reptile fence on individuals who gained accessed to the highway via fence-gaps. Reptiles may be forced to spend an increased amount of time on, or adjacent, to the highway in search of a corresponding gap in the fence on the other side of the road (Wilson & Topham 2009). Considering that that I found no reduction in turtle abundance on the road and an increase in both snake and turtle mortality, I conclude that a non-continuous flexible, plastic fence is not effective at preventing, or reducing, the threat of road mortality for reptiles. The ineffectiveness of the exclusion structure tested in my study should not be generalized to all fencing; there have been many studies that document high success rates with various styles of fencing (Dodd et al. 2004; Aresco 2005). This begs the question, why was the particular fence in my study not effective, when other exclusion structures have been?

Close examination of the fence revealed a suite of issues that rendered this style of exclusion structure ineffective, including the material, placement, and installation process. After the first winter post-mitigation, a total of 115 reptile-sized (or larger) gaps were located along the 6 km of sectional fencing (covering 3 km of highway). The fence material was easily ripped and torn, and in many areas wash-outs occurred because of improper installation. These issues created large areas with little to no barrier to prevent road access. Furthermore, during the spring melt up to 30% of the fence was semi-submerged, which allowed reptiles to climb over the 0.05-0.15 m of fence protruding above the waterline. The fence was no longer at the recommended height of 0.6 m for an effective barrier for reptiles (Woltz et al. 2008). Between areas that were deliberately not fenced and the many unintentional gaps, approximately 2/3 of the mitigated area was permeable, and the distance between locations of reptiles on the highway (N=91) to the closest known gap in the fence averaged 38.3 ± 4.2 m. This is a relatively small distance for these animals to move between considering their spatial ecologies (Ernst and Lovich 2009; Rowell 2012). This finding suggests a substantial association between the location of reptiles found on the highway and the closest location to an obvious means to breach the exclusion structure.

A solution to many of the problems I observed with the fencing design at my study site would be to use more durable materials, and ensure better placement. Plastic fencing is prone to rips and tears, quickly degrades over the short-term, and requires regular maintenance (Aresco 2005). Also, both plastic and metal mesh fences are easily climbed by many reptile species (Aresco 2005; Griffin & Pletscher 2006). High-water levels and drainage must be taken into consideration so that the threat of washouts and flooding are minimized, as exposure to water will degrade or destroy a plastic or metal fence. Roads are built to be long-lasting structures in that landscape, and the mitigation measures for roads should be equally long-lasting. A better option for fencing would be a concrete or steel gravity wall fitted into the sloped gravel between the shoulder and ditch, which would provide a solid long-lasting barrier. Effective barriers such as this have seen reduction in reptile road mortality up to 93% (Dodd et al. 2004), making these exclusion structure far more biologically effective. There may be a higher initial cost for the installation of a more durable, permanent exclusion structure that is incorporated into the road design. Yet, over the long-term, this mitigation option may be far more cost-effective compared to the intense annual maintenance (Aresco 2005), that may be up to 100% the installation cost for geotextile fencing (Brown & Schueler 1997).

Effectiveness of the population connectivity structures

The ecopassages were minimally effective, and likely would have been more effective if the fencing was functioning properly. The ecopassage monitoring methods (*i.e.*, wildlife cameras, automated PIT-tag reader, and haphazard encounters) observed that 4 reptile species used ecopassages. However, the number of individuals documented within an ecopassage (N=14), was much lower than the number of individuals found on the highway during the same timeframe (N=127).

A common concern regarding crossing structures is the potential for them to become prey-traps (Little et al. 2002). I observed no predation events within the ecopassages, yet of the observed individuals in the ecopassages, 22.3% were known reptile predators such as: herons, raccoons, minks, and coyotes (Ernst and Lovich 2009; Rowell 2012). By contrast, reptiles only accounted for 2.8% of observed individuals within the ecopassages. Thus, at their current level of use, the ecopassages in my study would be highly ineffective for a predator to use as a hunting location. Instead, it is most likely predator presence in the ecopassage is simply road crossing of these individuals. Similar findings refuting the prey-trap hypothesis have been noted for both small and large mammals (Little et al. 2002; Ford & Clevenger 2010).

The radio telemetry study of Blanding's and snapping turtles established that 20% of the radio-tagged individuals had home ranges that overlapped with the highway. Post-mitigation all of the radio-tagged turtles that crossed the highway were not in close proximity to an ecopassage during the event. I speculate that it is likely that the crossing occurred through a drainage culvert that had been incorporated into the mitigation fence (Fig. 1.8), based on location of the turtles before and after crossing; however, it is possible that gaps in the fencing were used as well. Regardless of how they crossed, it was seen that these individuals had critical habitats (*i.e.*, nesting and overwintering sites) and seasonal habitats (*i.e.,* basking and foraging sites) on both sides of the highway. The dispersal distance, the square root of an animal's home range and distance thought to be within an animal's ability to cover during movements (Bissonette & Adair 2008) allows us to estimate appropriate distance for ecopassage placement and fencing lengths. For these two turtles species the dispersal distance averaged 667 m, indicating that the current ecopassages at my site, placed 400-600 m apart, were appropriately spaced. Thus, distance between the culverts should not have been a factor which caused their low use. However, when planning the placement of ecopassges for multiple target species it is important to ensure that either all target species have comparable dispersal distances, or use the lowest dispersal distance, as to not exclude an entire species from gaining access to ecopassages. Exclusion structures should also extend beyond the dispersal distance for any suitable habitat and buffer zone for target species. Spatial ecology of an organism needs to be considered when deciding on placement of ecopassages. Placement should not only be present in presumed ideal habitat areas (*e.g.*, ecopassages only contained within the areas where a wetland abuts a road). By extending the exclusion structures outside of the natural spatial dynamics of a given species (*e.g.*, if an organism's dispersal distance is 600 m, then the exclusion structure should extend a minimum of 600 m on either side of the culvert), possible circumnavigation around the exclusion structure will be likely be prevented – a factor that is crucial in determining the success of a connectivity structure.

In my study, turtles were less willing to use the ecopassage, and were twice as likely to refuse using an ecopassage as they were to use it. This was significantly different from results obtained in arenas (Woltz et al. 2008; Paulson 2010). Such differences may be due to the fact that I tested ecopassage use beneath a live highway with the sights, sounds, and smells of traffic. The substrate (*e.g.*, soil, gravel, water, vegetation, etc.) also varied throughout the active season, which may have provided another potential for difference in ecopassage use; however, the seasonal variation of substrate is representative of real world situations. My findings thus provide a more realistic understanding of a turtle's response to an ecopassage. The likelihood of usage observed in arena tests may have been driven by the experimental design in which the turtle was only provided a single option, the ecopassage (simulating a road crossing), and was given no opportunity to escape (Paulson 2010). If conservation biologists are to achieve rates of ecopassage usage as high as those reported for arena studies, the associated exclusion structure needs to remove any other crossing option (*e.g.*, crossing over, under, through gaps in the fence, or circumnavigation around the fence). Without other crossing options, and given enough time (72% of painted turtles were willing to use a crossing structure when given 90 minutes; Paulson 2010), turtles should be far more likely to use an ecopassage. Therefore, the effectiveness of population connectivity structures relies on the effectiveness of the exclusion structures, demonstrating the importance of complete and secure fencing to bolster reptile use of ecopassages.

The genetic samples collected during my study will be used to create pre-mitigation baselines for future research on the effects of mitigation structures on population genetics. The negative effect of roads on a population's genetics has been identified as a serious concern to amphibians and mammals; however, such effects have yet to be examined in reptiles (Holderegger & Di Giulio 2010; Beebee 2013). My creation of a genetic baseline will allow for future studies that could examine potential shifts in the population genetics of multiple reptile species in response to mitigation measures. It will be studies such as this that will definitively determine the effectiveness of population connectivity structures.

Conclusion

Conservation decisions need to be supported by rigorous science, and it is crucial for highway designers and wildlife managers to thoroughly test the effectiveness of mitigation measures (Lesbarrères & Fahrig 2012; van der Grift et al. 2013). As global biodiversity decreases (McKee et al. 2004), and threats to animal populations are identified, we must strive to increase our level of protection for rare and imperiled species beyond current norms. We need to understand that mitigation is more than a political mandate satisfying a piece of legislation, it is a means to create infrastructure that directly reduces the negative impact of development on species (Dodd et al. 2004). Mitigation measures should be designed to last over the long-term (*i.e.*, the lifespan of the road) and require minimal annual maintenance. Materials used for exclusion structures needs to be enduring (*e.g.*, concrete gravity walls, solid steel barriers), should be incorporated into highway engineering and provided the same level of scrutiny given to road construction. The effectiveness of the population connectivity structures relies on the effectiveness of exclusion structures. Although I observed some use of ecopassages (via wildlife cameras and PIT-tag readers), it occurred at low rates, particularly when other crossing options existed. Furthermore the radio-telemetry study indicated that much like the results of the behavioural trials using ecopassages, turtles were occasionally willing to use a drainage culvert as a crossing structure; however, this was likely due to the culvert's specific placement (aligning with an open channel used as a movement corridor) rather than a desire to use the culvert. Although the success of the ecopassages tested was low, ecopassages can be effective when the associated exclusion structures are working (*e.g.*, Payne's Prairie Ecopassages; Dodd et al. 2004). Studies examining the effectiveness of mitigation for imperiled species must also examine long-term effects (*i.e.*, population genetics). I collected pre-mitigation tissue samples to establish genetic baselines for 11 species of reptile, allowing for future studies to fully determine if these population connectivity structures are effective. If care is taken to properly assess the effectiveness of mitigation, and steps are taken to adaptively manage the negative impacts of roads, then there will be not only benefits to imperiled species, but also to the overall ecosystem and human health alike (Brooks et al. 2006).

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Tables

Table 1.1 Number of reptiles, alive (AOR) and dead (DOR), observed on the road between samples periods (Before, After) and sites (Control, Impact).

	Before				After			
Taxa	Control		Impact		Control		Impact	
	AOR	DOR	AOR	DOR	AOR	DOR	AOR	DOR
Turtle	20	121	18	39	15	108	8	49
Snake	41	131	26	55	34	261	7	63
Total	61	152	44	94	49	369	15	112

Figures

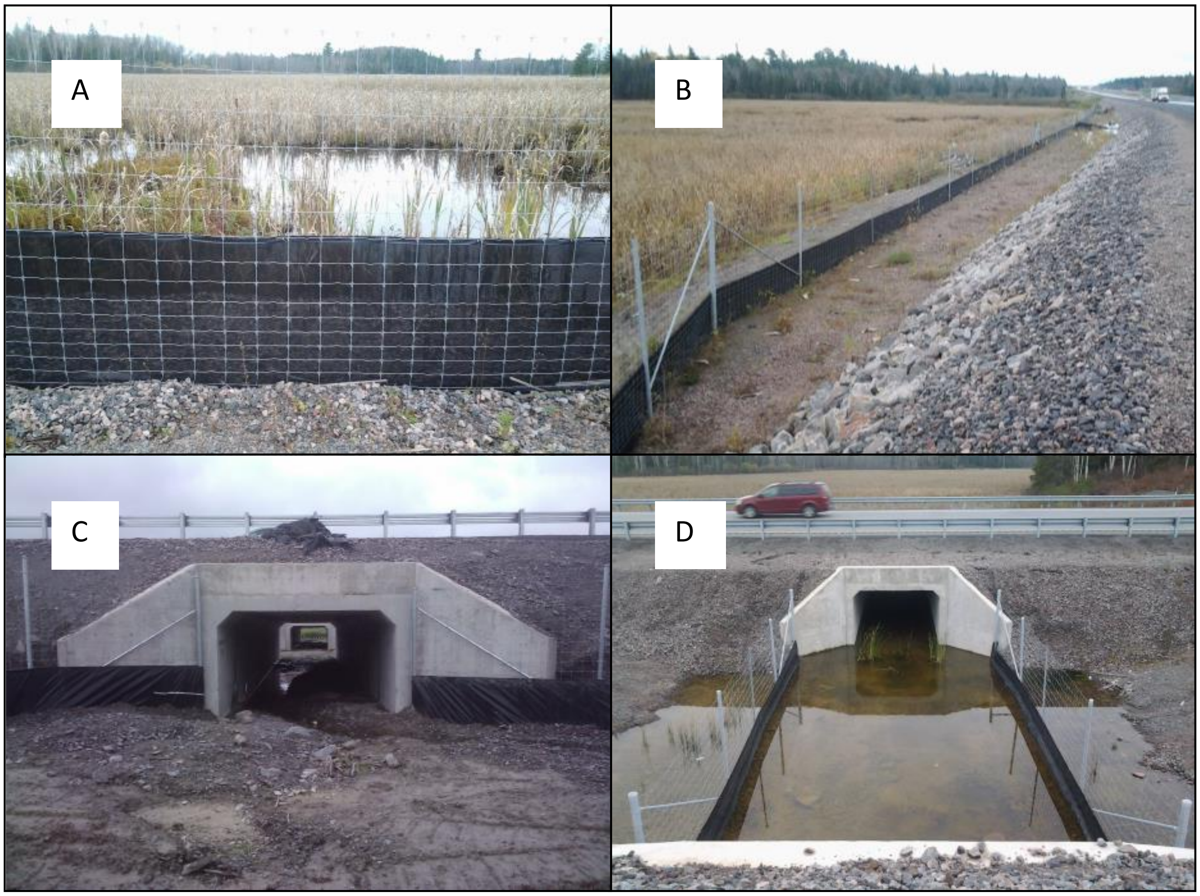

Figure 1.1 Mitigation measures completed during the fall of 2012 along Highway 69/400 in central Ontario, Canada. These measures include reptile fencing consisting of a heavy gauge plastic geotextile extending 0.8 m above- and 0.2 m below-ground with a 0.1 m wide lip running perpendicular underground (A). The fence was affixed to a 2.3 m tall large mammal, wire fence and was installed in areas believed to pose a risk to reptiles (B). Three ecopassages were built within the fenced area and each consists of two 3.4 m (width) x 2.4 m (height) x 24.1 m (length) concrete box culverts (C), separated by a 15.3 m gap for increased light (D).

2012.

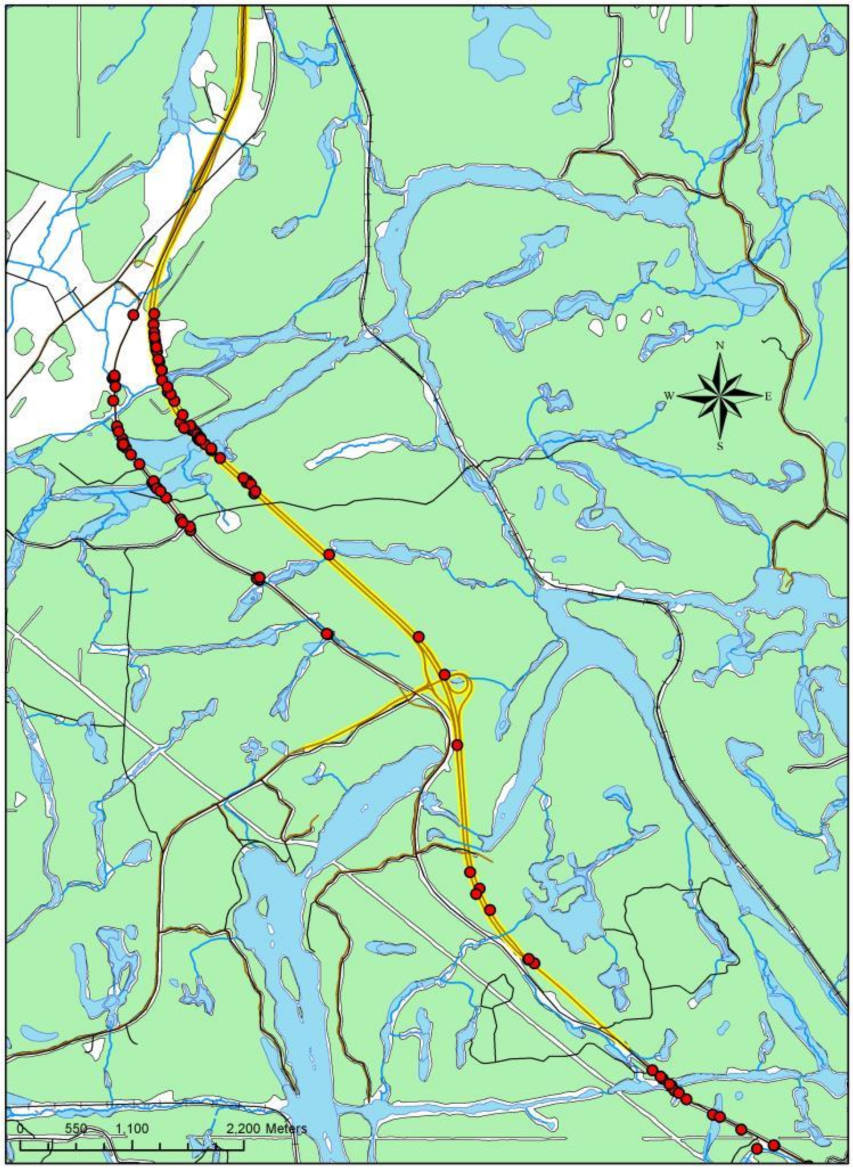

Figure 1.2 Location of reptiles (red circles) found on the road during the Before period (pre-mitigation) at the impact site; yellow lines represent the new alignment of Highway 69 opened 6 June 2012 and mitigation was not completed until after the field season during the autumn of 2012.

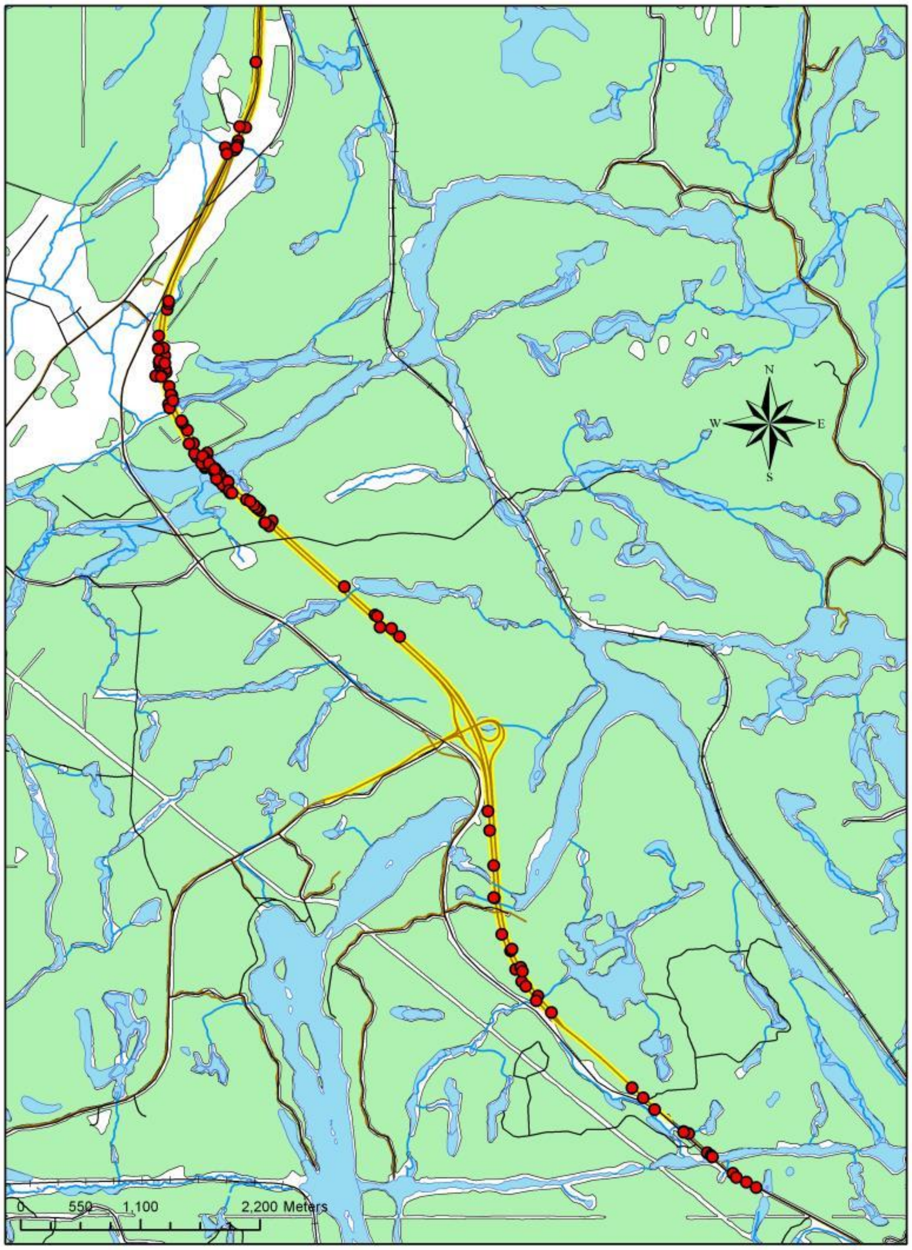

Figure 1.3 Location of reptiles (red circles) found on the road (yellow lines) during the After period (post-mitigation) at the impact site.

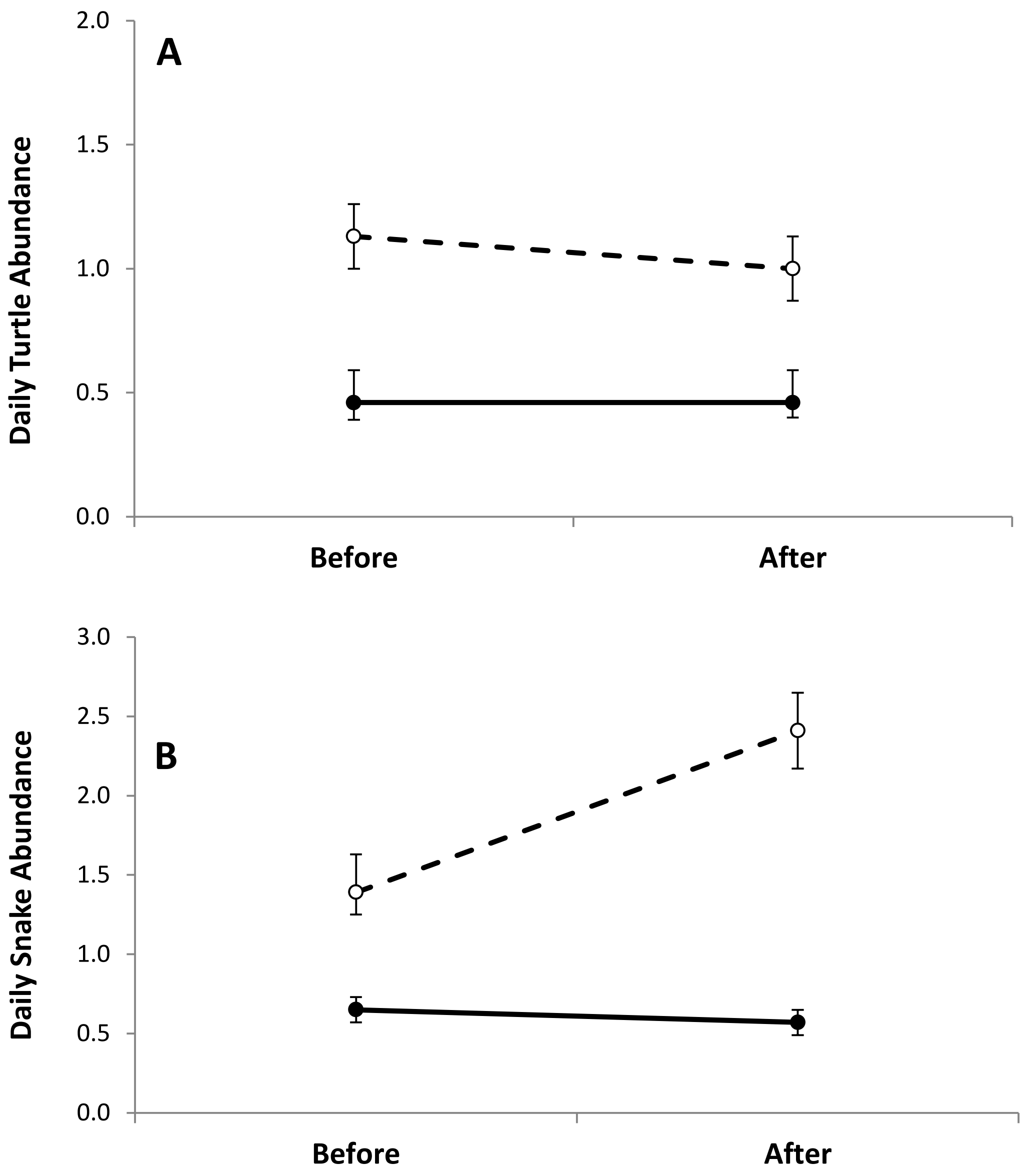

Figure 1.4 Daily abundance of reptiles on the highway for each survey period (Before and After) did not differ for turtles (A), but did differ for snakes (B) when considering survey sites (Impact (●) and Control (○)). The parallelism between the solid and dashed lines visually represents no significant interaction between site and period for turtles (A; GLM $z_{488} =$ -0.05, P = 0.57), while this interaction was significant for snakes (B; GML $z_{488} =$ 3.60, P < 0.01).

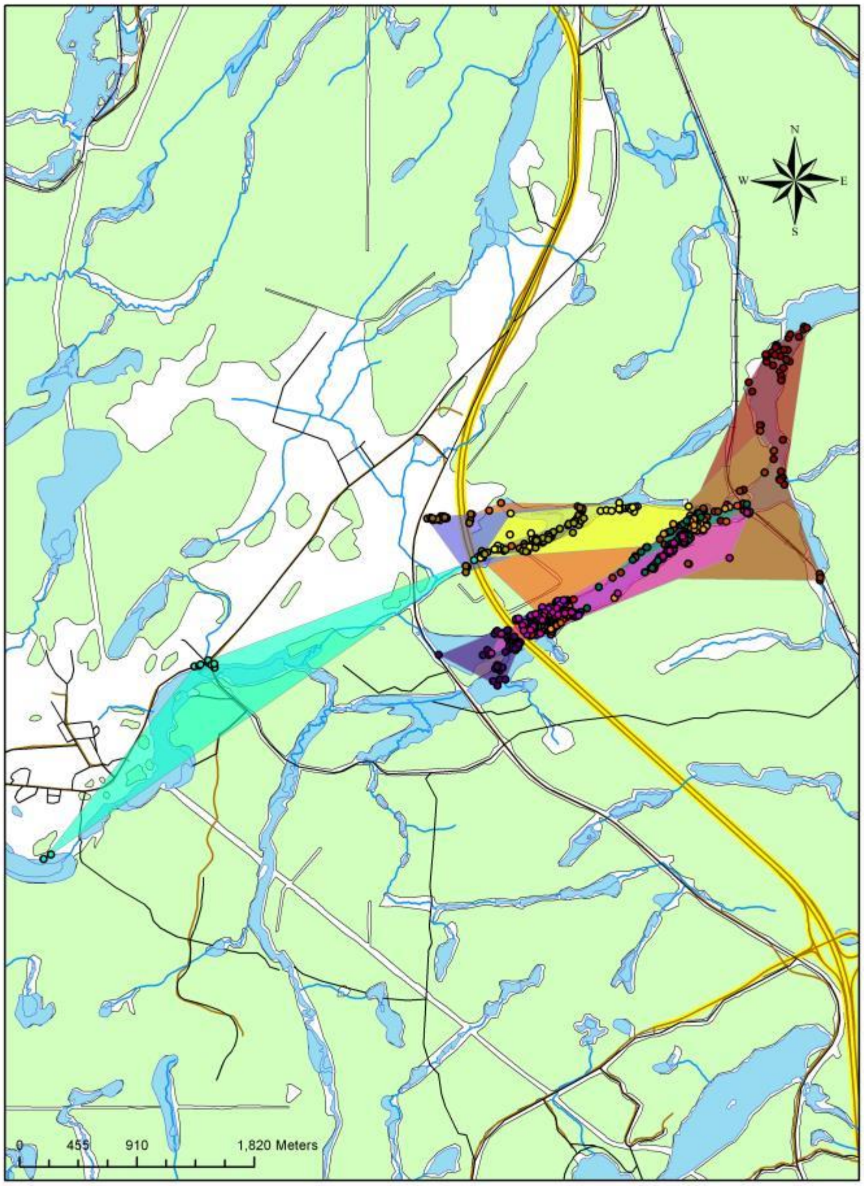

Figure 1.5 Map of Blanding's and snapping turtle home ranges pre-mitigation (2012), individual's locations marked with different colour points, and home ranges with corresponding minimum convex polygons. The yellow line indicates the new alignment of Highway 69, however during 2012 this aliment was under contraction through the active field season.

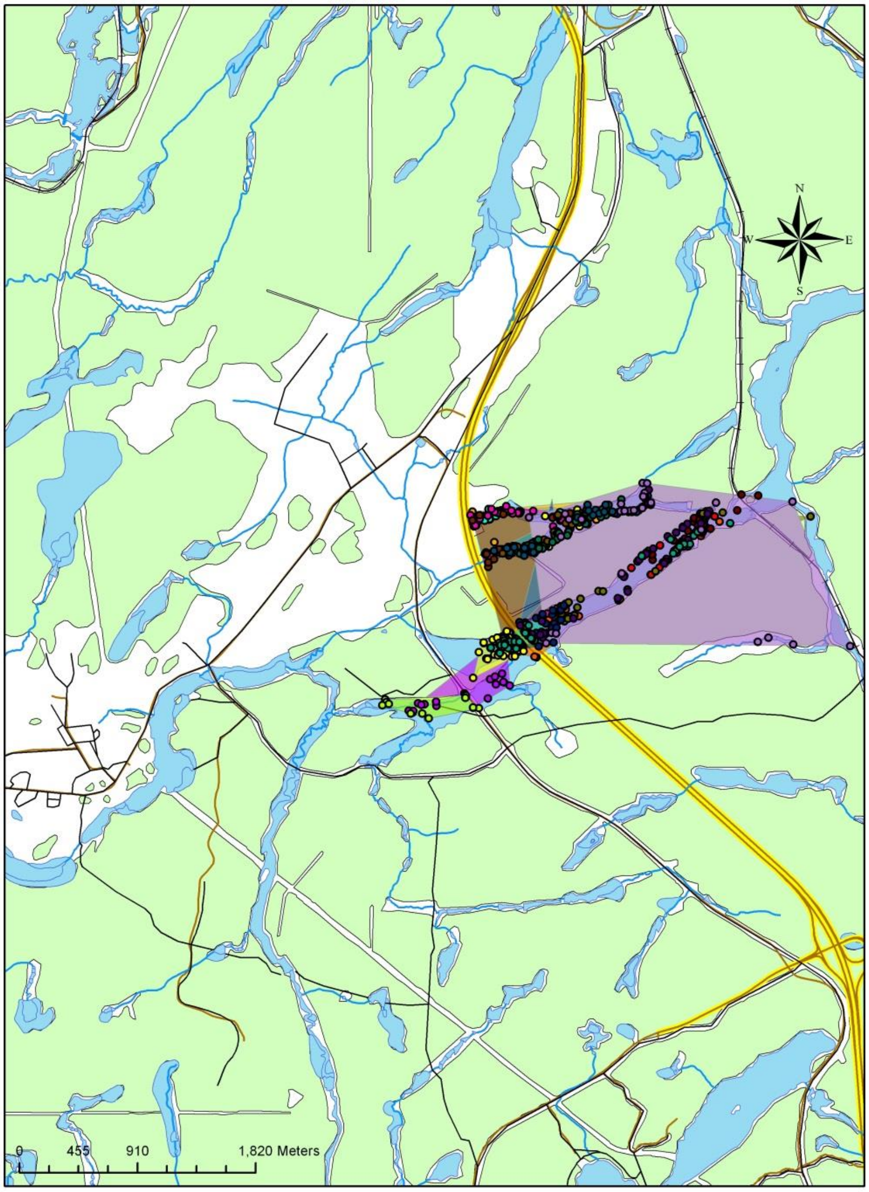

Figure 1.6 Map of Blanding's and snapping turtle home ranges post-mitigation (2013), individual's locations marked with different colour points, and home ranges with corresponding minimum convex polygons. The yellow line indicates the new 4-lane alignment of Highway 69, shifted approx. 600 km east of the old alignment at Sheppard Lake.

Figure 1.7 On 28 June 2013, an adult female snapping turtle was captured after crossing the reptile fence (the black fabric extending 10-15 cm above the water), which due to an increase in water height is not at the recommended minimum height for reptile fencing along roads (60 cm; Woltz et al. 2008).

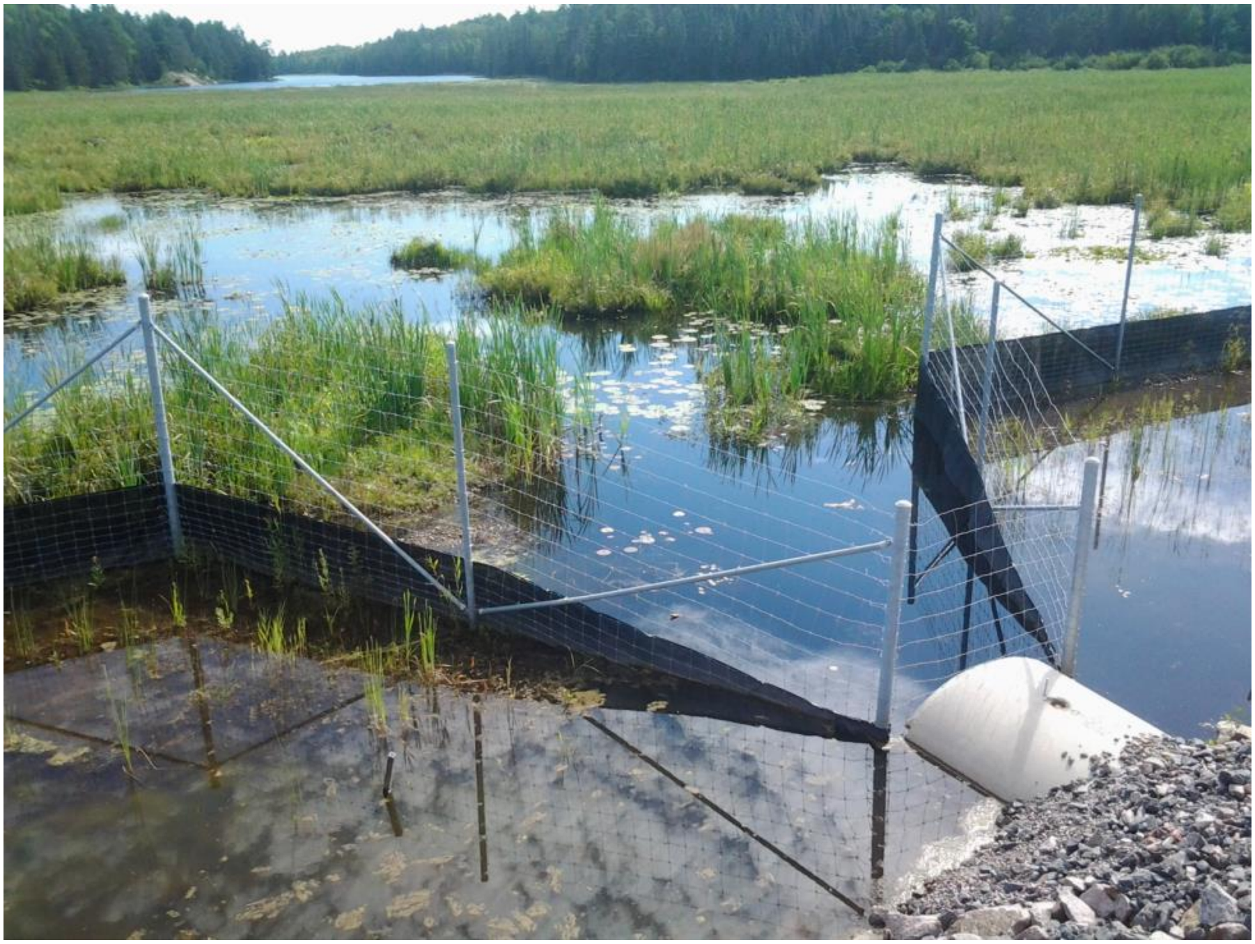

Figure 1.8 The drainage culvert located between ecopassages on Sheppard Lake, Ontario. Based on telemetry data, this culvert facilitated most of the road crossings (N=10) of radio-tagged turtles.

Chapter 2

Looking beyond the road:

Using a novel technique to measure corticosterone in claws to examine stress levels in painted turtles (*Chrysemys picta*) living around a major highway

Abstract

Conservation biology regularly incorporates multiple disciplines to expand its ability to identify threats to populations and develop the means to mitigate such threats. Road ecology is specialized, conservation-based research that examines interactions between wildlife and roadways. Within this field, the threats of direct road mortality and fragmentation caused by the road have received much attention, yet more research is needed to understand the indirect physiological effects of roads on wildlife. Chronic physiological stress lowers immune function, and can affect reproductive rates and life expectancy. Reptiles are globally in decline and many of these population declines have been associated with roads; however, it is unknown if chronic stress associated with individual responses to road infrastructure and traffic is also a substantial threat to population viability. In partnership with Dr. Mastromonaco (Toronto Zoo), I successfully extracted reliable measures of corticosterone (CORT), a known, commonly-used biomarker for physiological stress, from claw clippings from painted turtles (*Chrysemys picta*) captured at three study sites (road-impacted site, control site, and verification site). CORT levels in claws are indicative of chronic stress levels in these turtles, as CORT is slowly deposited over the long-term during claw growth. There were no differences in the level of CORT between turtles of road-impacted and control sites, nor a relationship between CORT and turtle body condition. However, male turtles had on average higher CORT levels than females. This study validates a novel approach for measuring CORT levels in a keratinized tissue as a measure of long-term stress for wild reptiles.

Introduction

In the face of dramatic biodiversity loss (Butchart et al. 2010; Hoffmann et al. 2010), conservation-based research is currently applied to a broad scope of disciplines (*e.g.*, reproductive biology, Wildt and Wemmer 1999; population genetics, Holderegger & Di Giulio 2010; thermal ecology, Monasterio et al. 2013; etc.), and typically includes topics that extend beyond those that simply address the most obvious threats to species loss (*e.g.*, habitat destruction, over-harvesting, emerging diseases, invasive species; Wilcove et al. 1998). Road ecology typically focuses on examining the threats of direct mortality on populations, as well as habitat and population fragmentation (Shepard et al. 2008; Clark et al. 2010). Although these threats are both prevalent and relevant, they are unlikely the only negative effects posed by roads to local wildlife populations. If we are to develop a complete understanding of threats to populations posed by roads, we must also look beyond the direct impacts of road itself. One such avenue of investigation resides in endocrinology, particularly in promoting the understanding of anthropogenically-sourced physiological stress (Newcomb Homan et al. 2003; Partecke et al. 2006; Van Meter et al. 2009). Several studies have examined road-related stress in birds (Crino et al. 2011, Morgan et al. 2012). However, reptiles, a group whose declines across Northern America are often associated with roads (23% in the USA; Wilcove et al. 1998, and 41% in Canada; Venter et al. 2006), remain absent from such investigation.

Physiological stress levels have been examined in reptiles based on reproductive cycle (lizards, Girling & Cree 1995; snakes, Graham 2006) and body condition (sea turtles; Jessop et al. 2004), and from stressors such as: capture/handling (sea turtles, Jessop et al. 2002; snakes, Bailey et al. 2009), invasive species (lizards, Trompeter & Langkilde 2011), and translocation (snakes, Holding et al. 2013; tortoises, Drake et al. 2012). The literature is scarce on the effect of urban environments on stress levels, with the exception of a few studies on desert lizards which examined baseline stress levels across a gradient of urbanization (French et al. 2008; Lucas & French 2012). Roads present a host of potential stressors in the forms of vehicle encounters, as well as sound, light and chemical pollution (Longcore & Rich 2004). Yet, the impacts these potential stressors have on the physiological state of reptile populations remains unknown.

Physiological stress is the result of an organism's response to negative stimuli, a stressor, through biochemical shifts away from homeostasis in preparation for the physical requirements related to the stressor (*e.g.*, priming the muscles during a flight or fight response; Romero 2004; McCarty 2007). Under normal circumstances, following the absence of the stressor the organism returns to homeostasis through secondary biochemical shifts (Romero 2004). This entire process is generally controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Romero 2004; Busch & Hayward 2009). Under normal conditions, acute stress would result in short-term reallocation of energy away from long-term physiological functions (*e.g.*, reproduction, growth, immune function); alternatively chronic stress may result in long-term physiological functions becoming disrupted or inhibited (Cabezas et al. 2007; Cyr & Romero 2007). From a conservation standpoint, understanding the relationship between anthropogenic stressors and chronic stress, and the associated implications to population health, is a crucial expansion of research that will aid in determining if there are indirect threats from anthropogenic structures to populations in decline (Busch & Hayward 2009).

Physiological stress is typically assessed by quantifying the levels of glucocorticoids (GCs, biochemicals produced during the activation of the HPA axis) present in the body (French et al. 2008). In reptiles, a common GC that is used in research is corticosterone (CORT; Sandor 1972). Traditionally, sampling CORT in reptiles has relied on blood plasma (French et al. 2008) or fecal sampling (Kalliokoski et al. 2012). Recently, methods have been developed to quantify levels of CORT in keratinized reptile tissues (snake sheds; Berkvens et al. 2013). In keratinized tissue, CORT is deposited over the long-term, and does not fluctuate over short time periods, like minutes to hours as it does in blood plasma (Romero & Reed 2003) or days to weeks as it does in feces (Berkvens et al. 2013). Instead, CORT is deposited during growth of a keratinized tissue, so samples represent long-term CORT levels, in turn accurately indicating levels of chronic physiological stress (Berkvens et al. 2013). Furthermore, sampling keratinized tissue is typically less invasive than collecting blood, and does not have the confounding effect of animal handling influencing the CORT levels measured, as is the case with blood (Berkvens et al. 2013). In this study, I developed and validated a novel technique to sample CORT non-invasively from turtles, using claws, a keratinized tissue that can be sampled in the field.

The objectives of this study were: 1) to develop a non-invasive, simple method to effectively determine chronic physiological stress in turtles, and 2) to use this method to determine if freshwater turtles living around major roadways are experiencing chronic physiological stress. If I am able to effectively measure levels of CORT from turtle claw samples, then the measures of CORT recovered from serial displacements, samples incrementally decreasing in concentration, from collected samples and synthetic samples should show a parallel displacement, resulting in a similar trend. Provided I could develop an effective means of determining long-term CORT levels in turtles, I will then examine the effect of roads on turtle CORT levels. If turtles living around highways are experiencing chronic physiological stress, then I should see higher levels of CORT in turtles living in close proximity to a major roadway as compared to those living farther from a roadway.

Methods

Sample collection

Three main study sites were used for sample collection: a road-impacted site (Highway 69, a major traffic corridor in central Ontario, Canada), a control site, (Neily Lake, Burwash, Ontario, Canada), and a validation site (Magnetawan First Nation, Ontario, Canada). The test site was a 4-lane highway connecting central and southern Ontario, with high level of annual reptile road mortality (Chapter 1). The test section of the highway was a construction site 4 years prior to testing, and had traffic flow for 2 years prior to testing. The control site, located 2.5 km from Highway 69, was located on a Department of National Defense (DND) property used as a firing range and training ground. Previously the property had been used as a prison farm between 1914-1973 and now has low use dirt roads running along the north shore of the lake. The validation site is a First Nation community in central Ontario bisected by Highway 69; samples from this site were not road-dependent and thus were collected from individuals found on and off the road. The samples from the validation site were used to test the effectiveness of the method, while samples from the road-impacted and control site were used to examine the effect of roadways on chronic stress in turtles. The midland painted turtle (*Chrysemys picta marginata*) was my model organism, due to its local abundance in both road-side (along Highway 69/400) and more natural habitats (70.2% of the turtles found along the highway were painted turtles; Baxter-Gilbert et al. unpubl. data, 2013). Alive and dead adult turtles were collected at the road-impacted by daily driving surveys (along 13 km of highway), and a daily walking survey (along 2 km of highway; see Chapter 1). At the control site, turtles were captured via hoop-traps, basking-traps, incidental encounters, and dip-netting from a canoe. Turtles were captured at the validation site using all of the capture methods described above.

Claw tips of both hind-feet (typically 8 individual claws) were trimmed from captured turtles using scissor nail-trimmers (Resco, Walled Lake, MI, USA). Care was taken to only remove the first 1-4 mm of nail (depending on wear), which prevented contamination of the sample from the blood vessel which runs along the center of the claw. Claw trimmings were then stored in labelled 20 ml scintillation vials (Fisherbrand, Leicestershire, UK) and stored at room temperature until processing (within 4 months of collection). Following claw sampling, turtles were sexed (adult male or female, unknown juvenile), weighed using a spring scale (100 - 2500 g model, Pesola, Barr, Switzerland). Morphometrics were measured with calipers (15 cm, Scherr-Tumico, China; 40 cm, Haglof Inc., Langsele, Sweden). Turtles were individually marked using a tapered file (Mastercraft, ON, Canada) and a notch code system (Cagle 1939) to prevent resampling, and released at their capture site within 8 hours. All field work involving animals adhered to the guidelines of the Canadian Council on Animal Care and an approved Laurentian University Animal Care Committee protocol (AUP# 2013-03-01).

Hormone Extraction

The lengths of the samples used for the validation study were measured with calipers, and ranged in length from 1.0-4.5 mm with a mean of 2.6 ± 0.1 mm (N = 168; Scienceware, Pequannock, NJ, USA). Samples were washed and crushed using modifications of methods described previously by Tegethoff et al. (2011) and Levitt (1966), respectively. In brief, claws were washed once with 1 ml distilled water, and then twice with 1 ml 100% methanol by vortexing for 10 sec. Samples were air dried, transferred to 2.0 ml cryovials (Corning Inc., Corning, NY, USA) and placed at -196°C for a minimum of 10 min in a liquid nitrogen dry shipper (Taylor-Wharton, Theodore, AL, USA). Frozen samples were placed in a steel cylinder and given several hard blows with a steel pestle. The crushed claw pieces were weighed using a Mettler Toledo balance (model AB54-S; ±0.0001 g; Mettler Toledo International, Inc., Columbus, OH, USA) and transferred to 7 ml glass scintillation vials (VWR, Mississauga, ON, Canada). CORT was extracted from the samples in 100% methanol using a ratio of 0.005 g/ml by agitating for 24 h on an orbital shaker (Montreal Biotech Inc., Kirkland, PQ, Canada) at 200 rpm. Samples were then centrifuged at 2300 g for 10 min and the extract was pipetted off into a new vial. The extract was dried in a fume hood and reconstituted in 150 µl EIA buffer solution (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per liter) resulting in a 1.13 to 16.53-fold concentration. Reconstituted samples were sonicated for 20 s in an Elmasonic waterbath (Elma GmbH & Co KG, Germany) before analysis.

Claw CORT values were quantified using modifications of an EIA described earlier (Watson et al., 2013; Metrione and Harder, 2011). Antisera were diluted as follows: goat anti-rabbit IgG (GARG) polyclonal antibody (Sigma-Aldrich, Canada), 0.25 µg/well; CORT (polyclonal CJM006, C. Munro, University of California, Davis, CA, USA), 1:200,000. The cross-reactivities of the antisera have been previously described (GARG and CORT: Watson et al., 2013; Metrione and Harder, 2011). CORT horseradish peroxidase (HRP) conjugate (C. Munro, University of California, Davis, CA, USA) was diluted 1:1,000,000. Standard solutions used were created with synthetic CORT (Steraloids Q1550; 39 – 10,000 pg/ml). The control consisted of laboratory stock of pooled fecal extracts obtained from spotted-necked otters (*Hydrictis maculicollis*) that was run at 65% binding. The reconstituted claw extracts were loaded and incubated on microtitre plates as described in Terwissen et al. (2013).

Validation Study

Analysis

Parallelism

Parallel displacements involve examining the relationship between a set of predicted values and actual test samples, and measuring the variance between them. The standard curve (created from synthetic stock) and a serial dilution (created from turtle claw extract) was used to detect immunological similarities between standard and sample hormones. A pooled sample of claw extracts was concentrated and serially diluted 2-fold from 1:65 to 1:4.1 concentrations in EIA buffer and run alongside the standard curve. Linear regression analysis was used to determine if there was a significant relationship in the % antibody recovered between the standard curve and serial dilutions of the sample extracts. A significance level of $\alpha = 0.05$ was used for all statistical tests.

Precision

Intra- and inter-assay coefficients of variation (CVs) were calculated to determine precision and repeatability. To control for intra-assay CV, only values from duplicates with < 10% CV were used as data based on real time monitoring of the CV of each duplicate run on each plate. Intra-assay CVs were further evaluated using a pooled extract at 50% binding loaded in different spots on the plate, and this was repeated three times. Inter-assay CVs were evaluated using a fecal extract control (65% binding; as noted above) loaded in duplicate on each plate.

Accuracy

Recovery of a known amount of hormone was calculated to examine possible interference of components within the extract with antibody binding. A pooled sample of claw extract was concentrated to 11-fold concentration. To 75 µl of pooled reconstituted extract, 75 µl of increasing concentrations of CORT standard were added in the range used for the standard curve. The concentrated pool was assayed alone to determine endogenous hormone levels. The percent recovery was calculated using the following formula: amount observed/amount expected x 100%, where amount observed is the value obtained in the spiked sample and amount expected is the calculated amount of standard hormone added plus the amount of endogenous hormone in the unspiked sample. Linear regression analysis was used to determine if there was a significant relationship between the hormone added and hormone recovered to assess assay accuracy.

Field study

<u>Analysis</u>

Differences in CORT levels extracted from claws were examined using an analysis of variance test (ANOVA) for the fixed effects of site (road-impacted, N=15; control, N=15) and sex (male, N=25; female, N=5). A linear regression was used to determine the relationship between CORT levels and body condition (N=18). Body condition was quantified using the residuals from a regression between turtle mass and maximum carapace length (Schulte-Hostedde et al. 2005; Litzgus et al. 2008; Rasmussen & Litzgus 2010). The residuals were evenly distributed across the range of carapace lengths in my study, thus I could assume the relationship was linear (Schulte-Hostedde et al. 2005). All statistical tests for this study were conducted in R statistical software (version 2.15.0, R Development Core Team 2012). All summary data are reported as means, followed by ± SE. All statistical analyses tested for interactions, but if the interactions were not significant only main effects were reported.

Results

Validation study

Serial dilutions of pooled turtle claw extract was significantly related to with the CORT standard curve ($R^2 = 0.95$, $P < 0.01$), and this indicates parallel displacement (Fig. 2.1). The recovery of known concentrations of CORT in turtle claw extracts was 92.8 ± 3.5% (χ ± SE). Inter-assay CV (variation between plates) was 3.3% at 65% binding. Intra-assay CV (variation within plates) was 5.6% at 50% binding. The measured hormone concentrations in the spiked samples correlated with the expected concentrations ($R^2 = 0.99$, $P < 0.01$; Fig. 2.2).

Field study

There was no difference in the amount of CORT between study sites ($F_{1,27} = 0.30$, $P = 0.59$). The amount of CORT found claws from turtles were 6.46 ± 0.69 ng/g and 7.69 ± 0.81 ng/g at the impact site and control site, respectively (Fig. 2.3). Females had significantly less CORT in their claws than males ($F_{1,27} = 6.08$, $P = 0.02$; Fig. 2.4). On average, females had 4.11 ± 0.56 ng/g of CORT in their claws, while males had 7.66 ± 0.56 ng/g. Finally, no significant relationship was noted between the residual index of body condition and CORT levels ($F_{1,16} = 0.062$, $P = 0.81$).

Discussion

Our validation study clearly showed that it is possible to extract reliable levels of CORT from turtle claws. This is a novel technique that can be used to simply and non-invasively collect reliable samples from turtles that reflect long-term CORT levels, and allows for the examination of chronic physiological stress. Interestingly, the average recovered amount of baseline CORT from claw samples fell within the range of the baseline amounts of CORT recovered from blood samples for other groups of reptiles (between 2-12 ng/ml seen in green sea turtles (*Chelonia mydas*), garter snakes (*Thamnophis sirtalis*), and marine iguanas (*Amblyrhynchus cristatus*); Moore & Jessop 2003). Recently the use of keratinized structures (*e.g.*, hairs, feathers, and snakes sheds) has provided valuable information into the long-term physiological stress in a number of mammals, birds, and snakes (Berkvens 2012). Expanding on this research, this method provides a new means for wildlife managers and conservation biologists to study stress, and examine this important proxy for population health in a variety of clawed reptile species using a minimally invasive, and easily collected sampling technique.

This study is also the first to examine if roads act as a chronic stressor for freshwater turtles. However, the findings did not support my hypothesis, as I did not find a difference in CORT levels between road-impacted and control sites. Two possible explanations for this result are 1) an unknown addition stressor at the control site, or 2) painted turtles may not have elevated stress levels due to highways. The control site is located adjacent to a small, low use dirt-road and a semi-active DND firing range and military training ground, allowing for occasional noise pollution and disturbance, albeit nowhere near the constant levels seen at the highway. The control site was also used a prison farm for most of the last century and Neily Lake may have encountered some chemical contaminant from the forestry and agricultural practices of that time. The potential for low level noise and remnant chemical pollution may have resulted in individuals at our control site have elevated CORT level from other (non-highway) sources. Although the possibility for unknown additional stressor exists, I feel the latter explanation (*i.e.*, painted turtles do not suffer from elevated stress levels due to highways) is more likely. My findings are similar to those from a study on tree lizards (*Urosaurus ornatus*) where no difference in baseline CORT levels was observed between populations living in urban and natural environments (French et al. 2008). It is important to note that both French et al. (2008) and my study used generally common reptiles as model organisms, likely due to their conspicuous nature and ease of capture. These species frequent anthropogenic environments and have not been noted to be in decline due to human presence. Within a conservation framework, this study is a conservative 'proof-of-concept' to our ability to easily examine reptile stress. Yet, more research is required on rare and threatened species in order to determine if they have a more severe response to anthropogenically-altered environments than more common species. A higher vulnerability to anthropogenic-influences may reflect their more severe population declines, in comparison to more common species, and increase understanding of the threats, including indirect ones, to these rare species.

Although understudied in freshwater turtles, body condition has previously been related to baseline levels of CORT in green sea turtles (Jessop et al. 2002). However, I did not observe a relationship between chronic stress and turtle body condition, in line with other sea turtle species (hawksbill turtles, *Eretmochelys imbricata*; Jessop et al. 2004). An alternative method for evaluating body condition, as opposed to using residuals from a regression between mass and body length, would be to examine immune function. Berger et al. (2005) examined immunocompetence of marine iguanas at varying levels of induced stress (both chemical and physical), determining that immune function was significantly decreased during periods of elevated CORT levels. Future studies should examine if painted turtles have a have a relationship between immune function and CORT levels to determine if immune function may represent a better proxy for examining the effects of elevated CORT levels. Furthermore, testing to determine whether variation in CORT levels recovered from claw samples correlate to more standard methods of measures physiological stress (*e.g.*, baseline blood or fecal samples, stress response using capture/handling techniques, etc.) should be done to further validate the relationship between long-term CORT levels in keratinized tissue to levels physiological stress.

Chronic CORT levels differed between sexes in this study, albeit there was a low sample size for females (N=5). The relationship between sex and baseline levels of CORT is well documented for reptiles; however, the findings are inconsistent. A difference in sex-specific CORT levels has been observed for some reptiles (Texas horned lizards, *Phrynosoma cornutum*, Wack et al. 2008; watersnakes, *Nerodia sipedon*, Sykes & Klukowski 2009; common wall lizards, *Podarcis muralis*, Galeotti et al. 2010), but not for others (freshwater crocodiles, *Crocodylus johnstoni*, Jessop et al. 2003; hawksbill turtles, Jessop et al. 2004; tree lizards, French et al. 2008). Overall, documentation of stress levels in freshwater turtles is largely absent from the literature. As turtles are the most imperilled group within the reptiles (Gibbons et al. 2000; Böhm et al. 2013), it is imperative that we increase our understanding of the relationship between stress, body condition, individual fitness, and overall population health (Wikelski et al. 2003; Bonier et al. 2009).

In the conservation of imperilled species it is vitally important to understand a population's physiological response to anthropogenic threats to biodiversity, such as habitat degradation, urbanization, and climate change (Newcomb Homan et al. 2003; Partecke et al.

2006; Van Meter et al. 2009; Satterthwaite et al. 2012). In understanding the physiological effects (*e.g.*, chronic stress) caused by human disturbance, conservation actions can work towards understanding the indirect threats which have until recently remained un-studied (Ellis et al. 2012). Examination of indirect effects of anthropogenic disturbance has broad implications for conservation biology. For example, when examining the effects of roads on imperilled species, it may be important to determine if population declines around roads are due to direct threats (such as high road mortality; Aresco 2005), indirect threats (such as chronic physiological stress), or a synergistic combination of both.

Global losses in biodiversity (Butchart et al. 2010; Hoffmann et al. 2010) require an ever-increasing need for comprehensive understanding of the threats posed to imperilled species. To understand and address the vast synergy of threats facing all forms of life, we are compelled to widen our perspective and look for threats beyond those most obvious. While the potential for anthropogenic disturbance to cause chronic stress in organisms remains understudied (Johnstone et al. 2012), I have determined a novel, simple, and non-invasive method to examine the levels of long-term CORT, providing a unique opportunity to detect if specific populations experience chronic stress due to human disturbance. Ultimately, conservation biologists need to identify and understand the presence, and diversity of threats to an imperilled species, before proper and effective mitigation can be developed and implemented.

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Figures

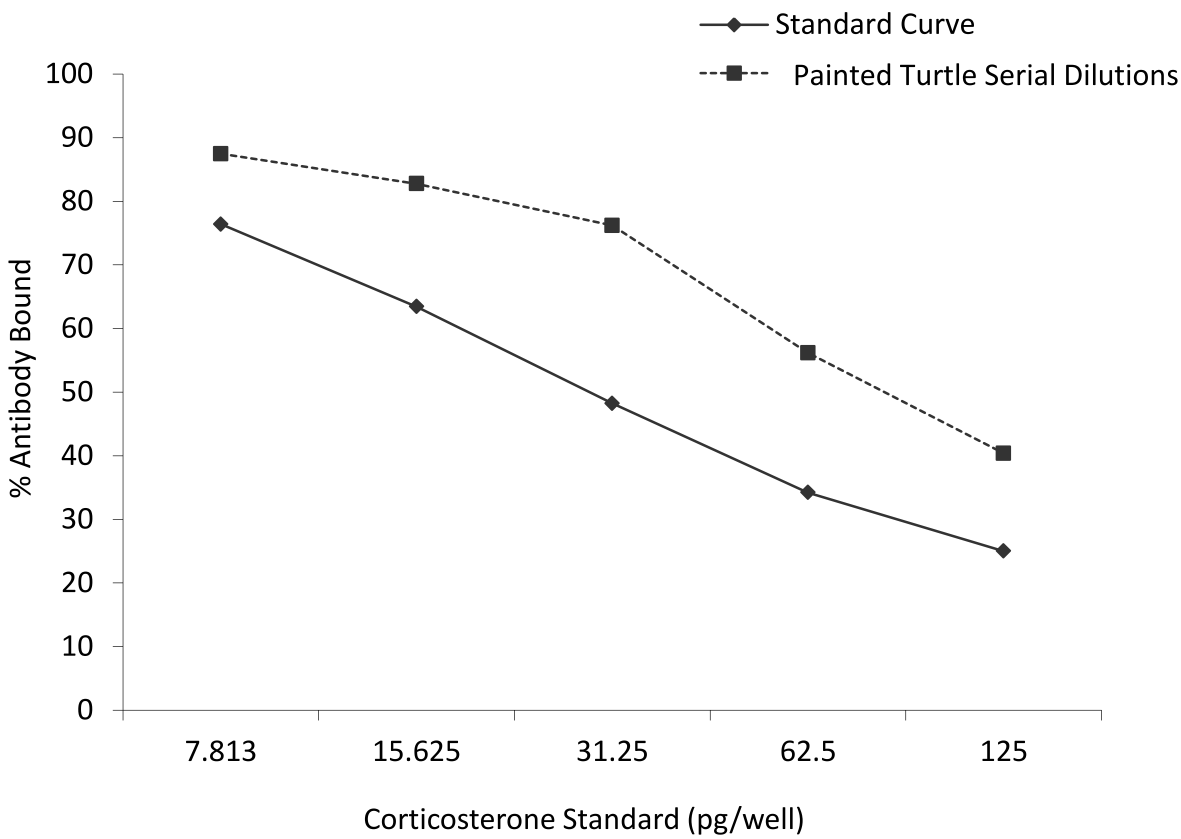

Figure 2.1 Parallelism between standard curve and serial dilutions of sample CORT extract. A significant relationship was found in the amounts of antibody bound to CORT between the painted turtle samples and that of the standard solution created from synthetic stock (R^2 = 0.952, $P < 0.01$).

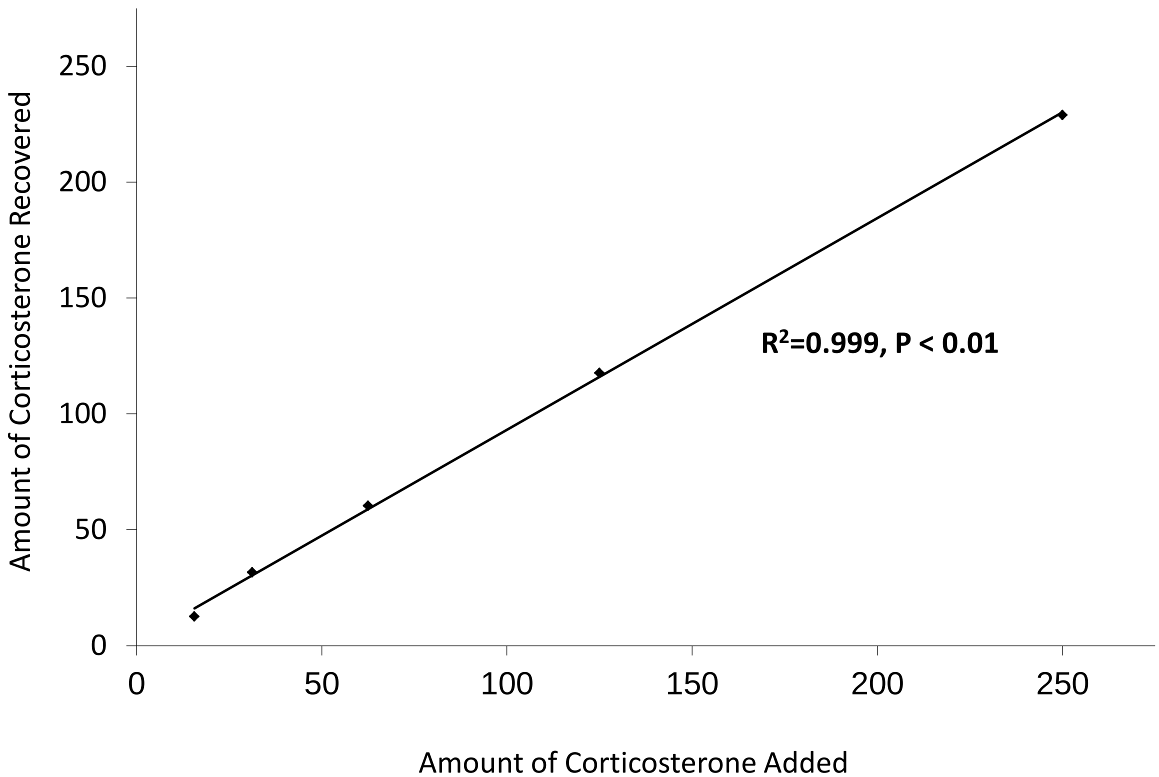

Figure 2.2 Recovery of exogenous corticosterone from turtle claw extracts, demonstrating a significant relationship between the amounts of CORT recovered from samples with varying amounts of spiked CORT ($P < 0.01$; glucocorticoids extract was from spotted-necked otter fecal samples), indicating our method closely followed the standard trend.

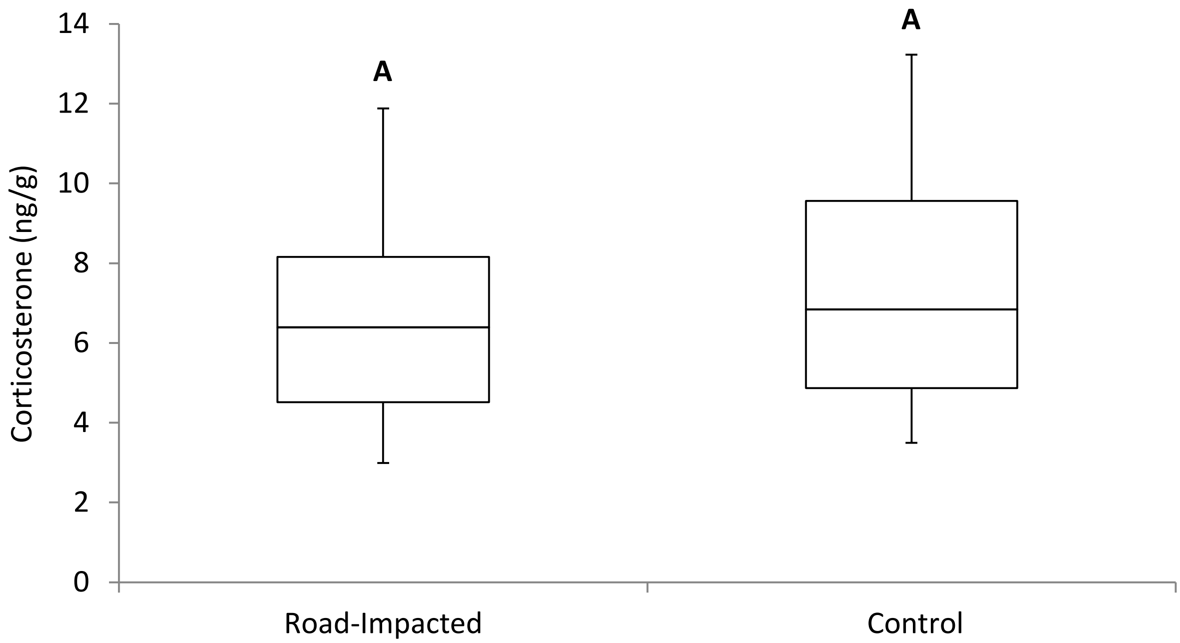

Figure 2.3 The average amount of corticosterone (CORT) recovered from claw samples collected from turtles living alongside roads (road-impacted) and at a more natural site (control). Common letters indicate no significant difference.

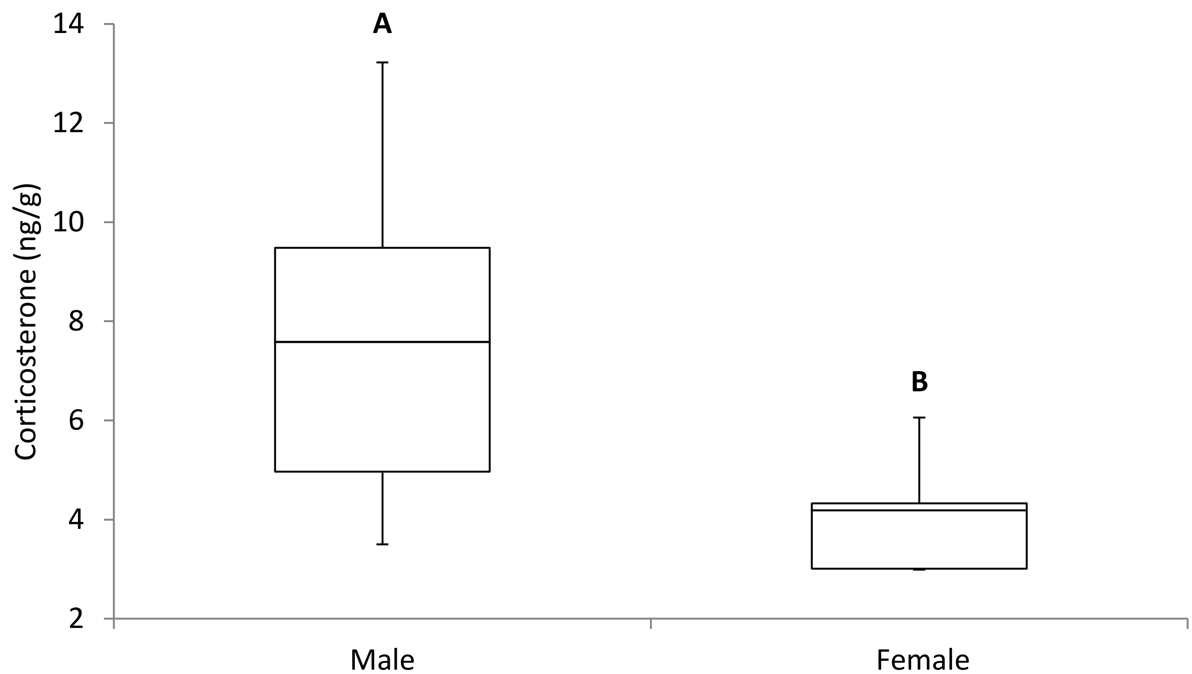

Figure 2.4 The average amount of corticosterone collected from claw samples for female and male painted turtles. Unique letters represent a significant difference.

Additional Research

My thesis has examined the effectiveness of mitigation measures for known threats (*e.g.*, road mortality, population and habitat fragmentation), as well as both developed and tested a novel method for examining subtle secondary negative effects of roads (*e.g.*, chronic physiological stress). For turtles, roads cause yet another threat: the creation of desirable, suitable nesting habitat adjacent to a known source of mortality (*aka.* gravel shoulders of roads; Steen et al. 2008). In order to mitigate the issue of turtles using gravel shoulders of roads as nesting sites, artificial nesting sites are often created away from the road with the intention of intercepting gravid females during nesting forays and providing an alternative to roadside nesting (Steen et al. 2006; Paterson et al. 2013).

During this study, a series of artificial nesting sites (ANS) were installed during the construction of the new Highway 69 expansion (at the impact site as describe in Chapter 1). The purpose of these ANS was to provide the turtles with an alternative to using the gravel shoulders of the road (Steen et al. 2006). The ANS were located between wetland habitat and the road, in an effort to intercept individuals heading to the road (Paterson et al. 2013). The ANS were monitored in 2013 using wildlife cameras (Pagnucco et al. 2011) to determine if gravid females were using these sites for nesting. Throughout the nesting season of 2013 (15 May to June 20) a total of 297 individual animals were documented at the ANS. Of these observations, 6% (N=16/297) were known nest predators (Ernst & Lovich 2009), and only a single reptile (0.4%; N=1/297; a snapping turtle, *Chelydra serpentina*) was documented. On 17 October 2013 the single turtle nest was noted to have an emergence hole, and upon uncovering the nest cavity 22 eggs shells (left from successful hatchlings that emerged), 1 living hatchling, and 3 unfertilized eggs were recovered.

Although use of the ANS was low, similarly to Paterson et al. (2013), this was likely attributed to the fact that a predominant nesting location was already established before the highway expansion. During my study, 11 nest sites were seen grouped in a 100 m^2 nesting site at railroad tracks located 1.8 km away from the highway and the ANS. Even though I only documented evidence of one turtle using the ANS site, this occurred shortly (within two years) after it was built, and successfully incubated a clutch of eggs. Thus, ANS is a very promising mitigation measure because use will most likely increase as gravid females wander across ANS during their seasonal movements. Also, the female that used the site in 2013 will most likely come back as females of multiple species are known to display nest site fidelity (Blanding's turtles, Congdon et al. 1983; painted turtles, Rowe et al. 2005; Mississippi map turtles, *Graptemys kohnii*, Freedberg et al. 2005). Further research on artificial nesting habitats is still required. However, the opportunity to offset turtle's selective nesting on roadsides may provide valuable population benefits by lowering the amount of turtles interested in using the roadsides, a benefit that could be further enhanced by the presence of an effective exclusion structure preventing road access.

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General Conclusion

The overall goal of this thesis has been to increase understanding of the threats caused by roads to reptile populations, as well as to assess the potential mitigation measures that could be used to prevent further population declines. In doing so I have rigorously evaluated the effectiveness of both exclusion structures, as well as population and habitat connectivity structures, and determined that more attention and effort must be taken to create long-term, permanent structures (*e.g.*, concrete or steel walls). This conclusion is based on the fact that the current method installed at my study site (*e.g.,* plastic, geotextile, and wire fencing) is extremely prone to failures (*e.g.*, rips, washouts, and improper installation and placement). My thesis highlights that failures in the exclusion structures causes, in turn, a reduction in the likelihood of use of population and habitat connectivity structures (*i.e.*, ecopassages) by reptiles. The best means to ensure ecopassages are effective is to ensure no other means of crossing exists via a durable, long-lasting exclusion structure. In my thesis I also developed, and tested a novel means of examining long-term stress, a potential indirectly-caused negative threat for species living around roads. My second chapter also highlights the importance of expanding the field of road ecology by integrating multiple fields of study. For example, in this chapter using a conservation physiology approach allowed me to assess the potential for a new host of indirectly-caused effect of roads on reptiles that have previously been unstudied. Overall, this thesis emphasizes the importance of further scientific research into means to protect wildlife from the direct threats caused by roads, and the expansion of our ability to detect and understand new, perhaps less obvious, threats roads pose to reptiles.

As global urbanization and the consequential demands for resources continue, there will be substantial losses to biodiversity. It should be our moral and ethical obligation, in opposition to our current role as the harbingers of destruction, to mitigate the negative repercussions of our species' progress, not only for the benefit of future generations of humans but for all life on this planet. Roads are one such form of anthropogenic infrastructure that persists across landscapes and bisects the natural world. Roads have a highly detrimental impact on wildlife populations, due to identified threats such as habitat and population fragmentation, but also a host of other indirect threats that may yet be discovered (*e.g.,* chronic physiological stress). Work in this thesis evaluated the effectiveness of current mitigation measures, and developed a means of examining the potential for unknown, and yet to-be-addressed, threats.

All too often the protection of ecosystems and the conservation of biodiversity are seen as a hindrance to traditional economic progress; however, as global biodiversity proceeds towards massive species loss and drastic environmental change, it is of grave importance that we put a higher value on the natural world. It is through rigorous scientific study that we can understand, ameliorate and mitigate the threats facing wildlife from ever-expanding anthropogenic threats. The need to create effective means to mitigate these threats is paramount, as is our ability to incorporate what we learn into adaptive management practices. It is important we understand that the mitigation of anthropogenic threats is more than a political mandate satisfying a piece of legislation, it is a means with which we can engineer infrastructure to directly reduce the negative impacts of development and human-progress on the natural world. Furthermore, we must not assume we are aware of every threat facing wildlife, and challenge ourselves to look outside the proverbial box. It has been humanity's intelligence and ingenuity that has imperiled this planet, and it is only through these same means that we may save it.